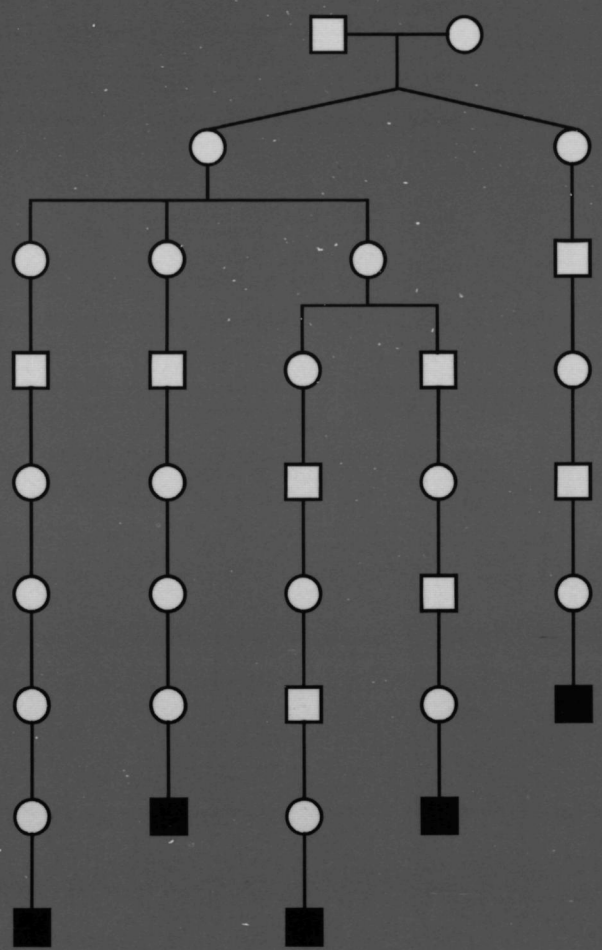
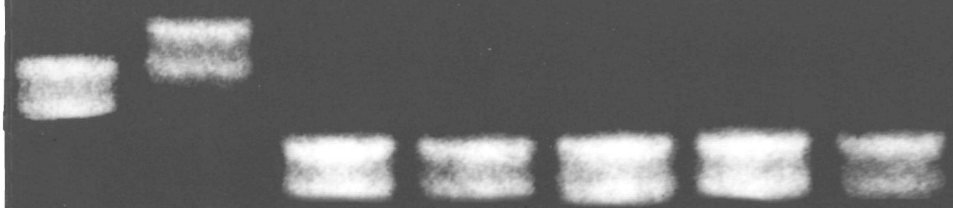
THE FRAGILE X SYNDROME

genetic and diagnostic aspects





A.P.T. Smits

The fragile X syndrome: genetic and diagnostic aspects

Frontcover: Linkage disequilibrium with CA repeat polymorphism at the DXS548 locus and the fra(X) gene in seven unrelated and five related fra(X) patients.

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The fragile X syndrome: genetic and diagnostic aspects

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen.

Proefschrift

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Ter herinnering aan mijn ouders en Sjaak
voor Ans, Femke en Marieke

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

Delineation of Genetically Determined Mental Retardation

Mental retardation, defined by an intelligence quotient (IQ) below 70, is a common and distressing disorder which occurs in approximately 1-3 per cent of the general population (Hagberg et al., 1983; WHO, 1986). Mentally retarded individuals can be grouped into two classes: (i) those that are severely affected (IQ <50) and (ii) those that are mildly affected (IQ 50-70).

Studies on the etiology of mental retardation have shown that the genetic contribution to severe mental retardation ranges from 25 to 52%, while for mild mental retardation about 7% may be caused by genetic factors (Gustavson, 1977; Roeleveld, 1992). Since a disproportionate excess (25%) of males was observed in various mentally retarded populations, it was considered likely that an X-linked pattern of inheritance was involved. Further support for the notion of an X-linked defect in a distinct subgroup of mentally retarded males was obtained by the identification of a number of nuclear families with an excess of male patients (Martin and Bell, 1943; Allan et al., 1944; Losowsky, 1961; Dunn et al., 1963; Renpenning et al., 1962; Snyder and Robinson, 1969; Lubs, 1969). Definite contributions to the delineation of X-linked mental retardation (XLMR) were made in the early 1970s by Lehrke (1972, 1974) and Turner & Turner (1974). Lehrke (1974) emphasized that XLMR may be responsible for 25 to 50% of the overall occurrence of mental retardation. Approximately one-third of these cases appeared to be due to the presence of the fragile X (fra(X)) syndrome (Turner et al., 1986; Webb, 1989).

Clinical and Social Phenotype of Fra(X) Syndrome

Studies on intelligence levels have shown that most affected fra(X) males are severely retarded (IQ 20-50) (Chudley et al., 1983; Kähkönen et al., 1983; Fryns et al., 1984, 1989; Veenema et al., 1987), but also higher functioning males do exist (Hagerman et al., 1994). Longitudinal observations indicate that IQ scores among fra(X) males decline later in life (Lachiewicz et al., 1987; Hagerman et al., 1989; Curfs et al., 1989; Fisch et al., 1991).

The most prominent physical features in affected adult fra(X) males (fig. 1) include an oblong face, large head circumference in early childhood, long and protruding ears, prominent jaw, extensible joints and macro-orchidism (Turner et al., 1980; Sutherland and Hecht, 1985; Hagerman et al., 1987). Many of these features become obvious in puberal males, although an

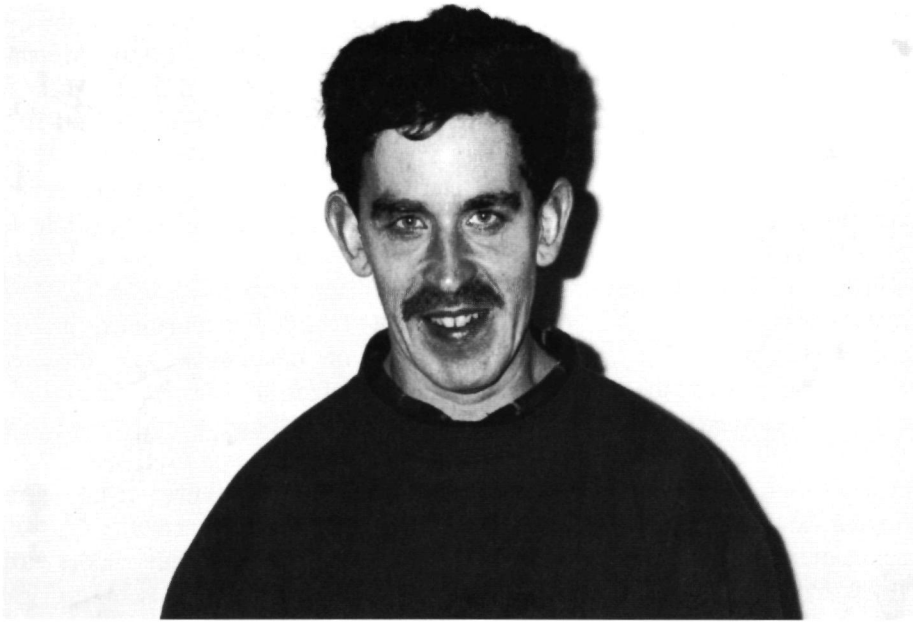

Fig. 1. Appearance of a typical fra(X) patient. Note the oblong face and prominent jaw.

extensive variability in physical and psychological expression can be observed, even between brothers (see also fig. 2) (Renier et al., 1983). The behaviour of fra(X) patients has autistic-like features, manifested as avoidance of eye contact and/or shyness even to relatives (Goldfine et al., 1985; McGillivray et al., 1986; Reiss et al., 1986, Borghgraef et al., 1987; Simko et al., 1989). In addition, hyperactivity and a characteristic speech such as stuttering and perseveration on words and phrases are striking features of these patients (Newell et al., 1983).

In general, heterozygous females are less severely affected than hemizygous males. These females display a broad range of dysfunctions and may be mentally normal, mildly affected, or even severely retarded (Kemper et al., 1986; Loesch and Hay, 1987; Veenema et al., 1987; Reiss et al., 1989; Borghgraef et al., 1990; Grigsby et al., 1990; Hagerman et al., 1992). About

Fig. 2. Two brothers with the fra(X) syndrome. Note extreme differences of facial appearances.

50% of these females are self-supporting in society (Cronister et al., 1991). The majority of fra(X) females also show a decline in IQ scores with increasing age similar as seen in male patients (Fisch et al., (1994).

Cytogenetic Detection of Fra(X) Sites

In 1969, Lubs found that the presence of a terminal constriction on the long arm of the X chromosome appeared to be associated with mental retardation (fig. 3). At that time, the importance of the association was not fully appreciated, as other laboratories were unable to reproduce his finding. In 1977, Sutherland discovered that the cytogenetic appearance of the so-

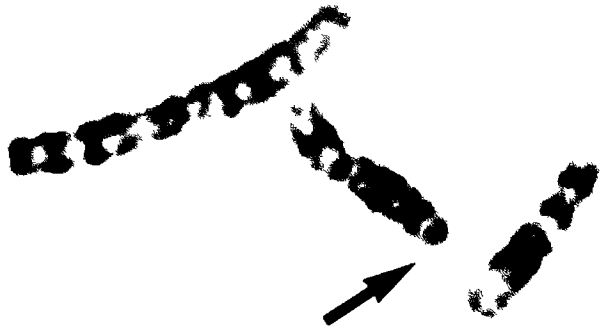

Fig. 3. Metaphase preparation of banded chromosomes. Arrow points to the fra(X) site on the X chromosome.

called fra(X) site was highly dependent upon the use of folic-acid deficient culture medium. This was followed by numerous reports in the early 1980s in which these findings were confirmed, and it was concluded that 30 to 50% of mental retardation with X-linked inheritance is indeed associated with the presence of the fra(X) chromosome (Herbst, 1980; Jacobs et al., 1980; Turner et al., 1980; Venter et al., 1981; Brookwell et al., 1982). However, the interpretation of the cytogenetic test results are not always straightforward. Even under optimal culture conditions the proportion of fra(X)-positive cells in affected individuals does not exceed 50%. Also the number of fra(X) sites does not show a positive correlation with the degree of the mental handicap. It is well documented now that, while the fra(X) site can be detected efficiently in clinically affected individuals, the cytogenetic detection rate of the mutation in mentally normal females is very low and zero in male carriers (Fryns and Van Den Berghe, 1982; Howard-Peebles and Friedman, 1985; Veenema et al., 1987; Smits et al., 1992).

Linkage Analysis Using Restriction Fragment Length Polymorphisms (RFLPs)

Since the early eighties, a number of investigators set out to find the molecular basis of the fra(X) syndrome. Genetic linkage indicated that the causative mutation must be located close to or in the fra(X) site itself. The first report of positive linkage with fra(X) associated mental retardation was published in 1983 by Filippi et al. using a glucose 6-phosphate dehydrogenase protein polymorphism. Camerino et al. (1983) reported the first linkage with a DNA marker (pFIX). Subsequent linkage studies in fra(X) families were focused mainly on the pFIX marker and a second, highly polymorphic, distal marker (pSt14). Although these latter two markers flank the fra(X) disease locus (Oberlé et al., 1986; Mulley et al., 1987), they show significant recombination with the fra(X) locus (Warren et al., 1985; Brown et al., 1988), severely limiting their applicability and accuracy in genetic counseling.

Obviously, more closely linked polymorphic markers were needed in order to make accurate carrier detection in fra(X) families feasible. Numerous additional markers were mapped within the Xqter region (Brown et al., 1988; Dahl et al., 1989; Oostra et al., 1990; Hulsebos et al., 1991; Rousseau et al., 1991; Suthers et al., 1991; van Oost et al., 1991; Riggins et al., 1992; Dreesen et al., 1993). These markers are summarized in figure 4.

An example of a DNA linkage analysis in a fra(X) family is given in figure 5. In this family the proximal probe pVK23B (Zz) with a recombination fraction of 1% and the distal probe pU6.2 (Ss) with a recombination fraction of 3%, were informative for carrier detection. Cytogenetic expression was present only in the mentally impaired individuals. In each of them the same "z s" haplotype was found. This haplotype was found also in the obligate carriers (III:5, IV:2 and IV:4). One further normal male carrier was identified (III:8) which implicated that his daughters were also at a high risk for carriership (99%). In this branch of the pedigree, linkage analysis revealed the presence of a carrier granddaughter (V:7). Prenatal diagnosis after chorionic villus sampling (CVS) was performed in 5 cases (IV:4,5,8,9,10) in this family using the CA-repeat RS46 (R2,R3), a highly polymorphic marker which is located about 150 kbp proximal to the fra(X) locus (Riggins et al., 1992), see also figure 4. In two of these cases (V:4, V:10) the allele at risk (R2) was found. This was confirmed by the cytogenetic observation of fra(X) sites in both villi samples and was consistent with the non affected individual III:8 being a carrier of the fra(X) mutation. This example illustrates that the development of closely linked polymorphic DNA markers has dramatically

increased the precision of post- and prenatal carrier detection and, as such, has contributed significantly to the feasibility of genetic counseling.

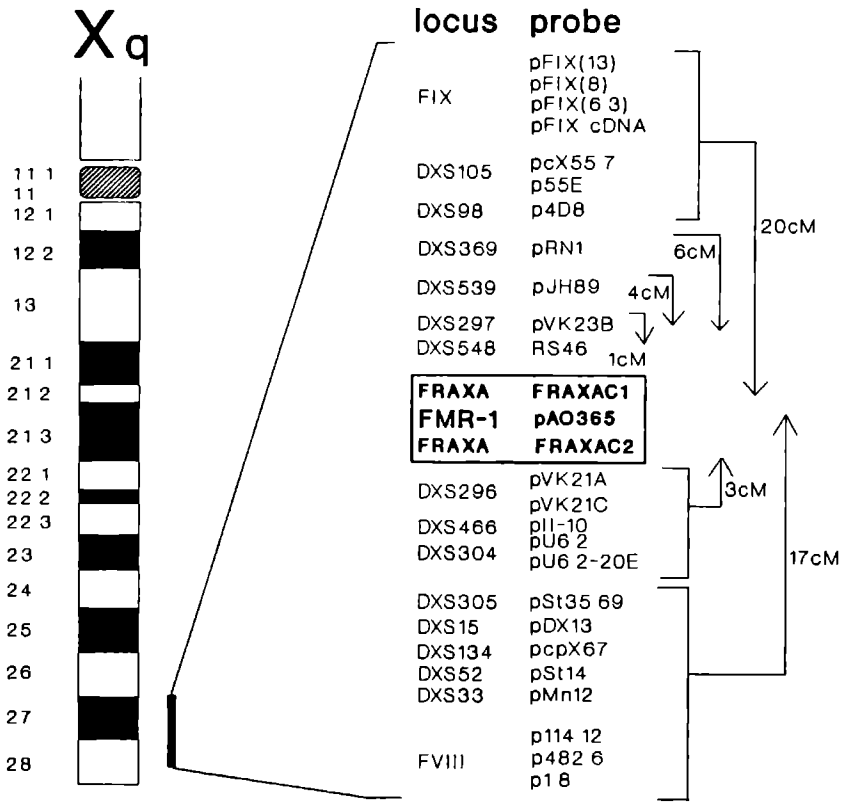

Fig. 4. Relative location and genetic distances for DNA loci physically and genetically mapped within the Xq27-q28 region.

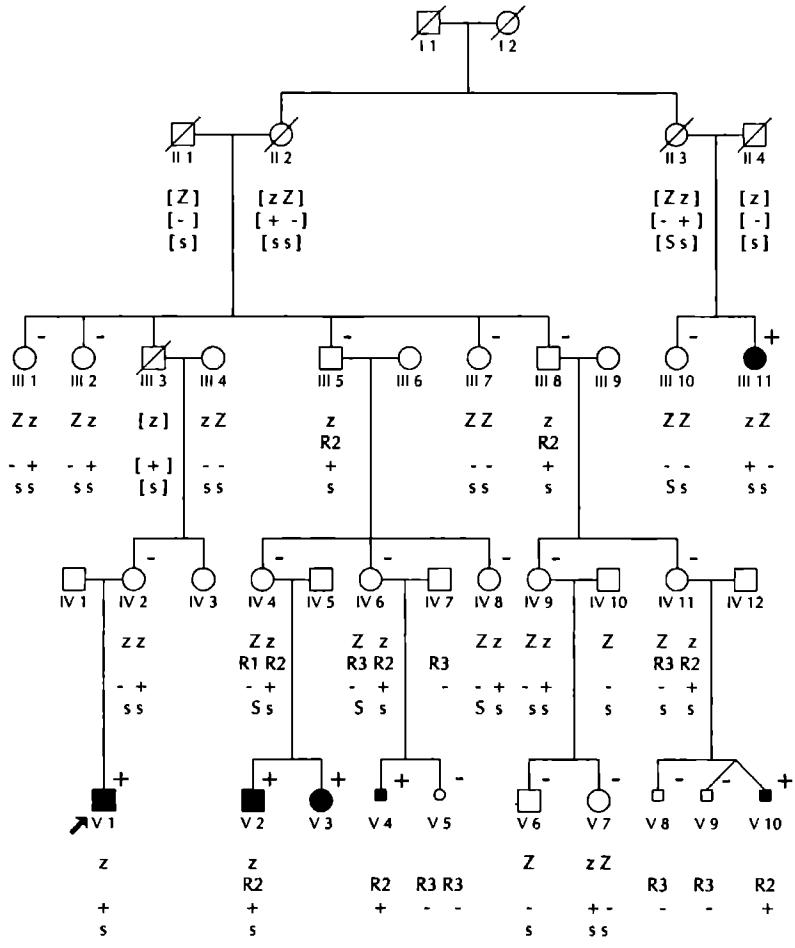

Fig. 5. Post- and prenatal carrier detection in a family with DNA probe pVK23B (Zz), DNA probe pU6.2 (Ss) and the CA-repeat RS46 (R2,R3). See figure 4 for the relative location of these markers. Absence and presence of cytogenetic expression is indicated by "-" and "+" , respectively, right above pedigree symbols. The linkage phase is indicated by "z+s" in which "+" indicates the presence or the inferred presence of the fra(X) mutation. The absence or the inferred absence of the fra(X) mutation is indicated by "-" in the haplotype notation. Affected individuals are designated by solid symbols. Pedigree symbols reduced in size represent prenatal tests and solid symbols of this latter group indicate the presence of the allele at risk. The probandus is marked by an arrow.

Cloning of the Fra(X) Locus and Carrier Detection Using CGG Repeat Length

Various techniques have been applied for cloning of the fra(X) locus. Vincent et al. (1991) and Bell et al. (1991) used pulsed-field gel electrophoresis and found a CpG island which exhibited abnormal methylation patterns in fra(X) patients. A YAC (yeast artificial chromosome) containing the region with the abnormally methylated CpG island was shown to span the fra(X) site by fluorescent *in situ* hybridization (Vincent et al., 1991). Oberlé et al. (1991) and Yu et al. (1991) performed Southern blot analysis using subclones adjacent to the CpG island and found evidence for unstable DNA. In fra(X) male patients no normal sized restriction fragment was present anymore but instead a multitude of different larger fragments were seen, presenting as a "smear". In normal transmitting males (NTMs) a small insertion (50 to 500 base pairs (bp)) was found.

Warren et al. (1987, 1990) generated a set of somatic cell hybrids via fusion of Chinese hamster ovary cells with lymphoblasts from a male patient with the fra(X) syndrome. After induction of fra(X) expression, hybrids were selected containing either the region proximal or the region distal to the fra(X) locus translocated to a hamster chromosome. These somatic cell hybrids appeared to be essential for subsequent mapping and cloning studies. Verkerk et al. (1991) identified a YAC which spanned the breakpoint in two reciprocal translocation hybrids. With a four-cosmid contig derived from this YAC, it was found that most of the breakpoints occurring in Warren's hybrids were clustered within a 5.1 kb EcoRI fragment. In this region also an abnormally methylated CpG island was found in fra(X) patients. These cosmids were, subsequently, used to isolate from a fetal brain cDNA library a cDNA corresponding to a gene that was designated *FMR1* (fragile X mental retardation gene 1). In the 5' end of the first exon of the *FMR1* gene a sequence was identified containing a CGG trinucleotide repeat. The previously mentioned "smears" appeared to be due to large expansions of the CGG triplet repeat.

More precise analysis of this repeat among normal individuals demonstrated frequent length polymorphisms, ranging from 6 to 50 repeats (fig. 6) (Kremer et al., 1991: Fu et al., 1991). In individuals with the fra(X) mutation the expanded CGG repeat can be classified in two main types (fig. 6).

(i) In nonpenetrant fra(X) carriers a restriction fragment length increase of 150 to 600 bp, corresponding to 50 to 200 CGG trinucleotide repeats, is

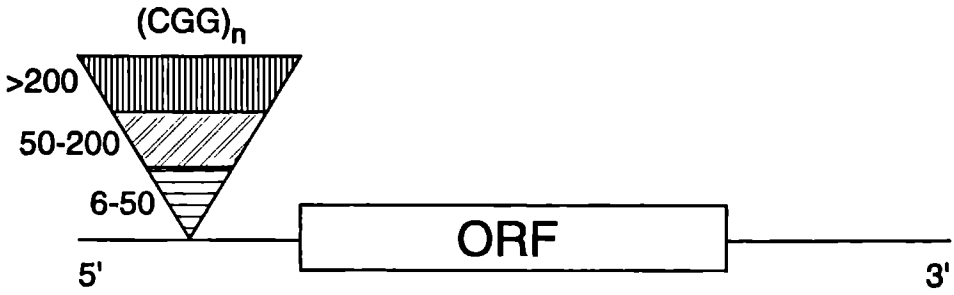

Fig. 6. Amplification of the CGG repeat leads to two main types of fra(X) mutations: a small insertion (premutation of 50-200) which does not cause mental retardation and a large insertion (full mutation of more than 200 triplets) with a high risk of mental retardation. In the normal population the repeat length varies between 6-50 (bottom part of the triangle).

found, which is termed a premutation sized allele. This fragment, next to a normal-sized fragment, is seen in daughters of NTMs. When a female carrier passes on her premutation chromosome the CGG repeat behaves extremely unstable and may undergo an increase in size to a full mutation (Fu et al., 1991; Oberlé et al., 1991; Heitz et al., 1992; Mulley et al., 1992; Yu et al., 1992). (ii) The full fra(X) mutation, which is characterized by a dramatic amplification of the number of CGG triplet repeats far beyond 200. This increase is somatically unstable and generates a multitude of restriction fragments ("smear") and leads to the characteristic phenotype of the fra(X) syndrome in males. On the other hand, female carriers of the full mutation may be either normal functioning or mentally affected (Rousseau et al., 1991; Mulley et al., 1992; Smits et al., 1992; Yu et al., 1992).

Scope of the Thesis

The objective of the studies described in this thesis was to gain insight into the enigmatic inheritance pattern of one of the most prevalent X-linked disorders associated with mental retardation: the fragile X (fra(X)) syndrome (fragile Xq27.3).

After an historical introduction in **chapter 1,** studies on the origin of the

fra(X) mutation within families are reported in **chapters 2 and 3**. The data indicate that the mutation rate for the fra(X) syndrome is low.

Incomplete penetrance of the fra(X) mutation occurring in females as well as in males, has important implications for genetic counseling and is the topic of the following three chapters. **Chapter 4** describes a refinement of the estimates of these penetrances, in particular with regard to the parental origin of the fra(X) mutation, the mental status of the carrier mother and the presence of a NTM in a given family. In **chapter 5** the direct estimate of the penetrance of the fra(X) mutation is compared with the estimate using a computer program based on the inheritance characteristics of sex-linked diseases. In **chapter 6**, the effect of the parental origin of the fra(X) mutation on both the cytogenetic expression and the CGG repeat length is reported.

At present, carrier detection for the fra(X) syndrome is carried out by direct detection of the CGG repeat. In order to evaluate the usefulness of this diagnostic test a validation is presented in **chapter 7**. In **chapter 8**, the sensitivity, specificity and predictive value of CGG repeat analysis is reported and discussed in relation to adequate genetic counseling in fra(X) families. **Chapter 9** describes a detailed study of a familial case with a full mutation in brothers without any clinical consequences. This exceptional family enabled us to assess relationships between CGG repeat length and methylation status of the 5' end of the *FMR1* gene, the transcription of the gene, the ultimate *FMR1* protein level, the cytogenetic fragile site expression and the clinical status.

Finally, in **chapter 10**, issues concerning carrier detection in high risk populations, in particular fra(X) families, are discussed.

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CHAPTER 2

HIGH PREVALENCE OF THE FRA(X) SYNDROME CANNOT BE EXPLAINED BY A HIGH MUTATION RATE

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SUMMARY

The overall prevalence of the fragile X (fra(X)) mutation, as determined by population studies, is approximately 1 in 850 (Gustavson et al., 1986; Webb et al., 1986). This prevalence suggests a very high mutation rate which, in turn, suggests that many patients have to represent sporadic cases. In order to obtain an accurate estimate of the proportion of sporadic cases, we performed genealogic, cytogenetic and DNA linkage studies as well as direct analysis of the CGG repeat in relatives of 84 fra(X) probands. We did not find any evidence for the presence of sporadic cases. In 11 probands consanguinity could be proven by the detection of common ancestors, in 5 related families up to 9 generations ago. In the other 6 related families the mutation could be traced back 4-6 generations. In 3 or more generation families we were able to demonstrate that half of the probands carried the grandpaternal fra(X) gene. These results imply that rather than a high mutation rate, both Normal Transmitting Males (NTM's) and mentally normal female carriers contribute considerably to the high prevalence of the fra(X) syndrome.

INTRODUCTION

The fragile X (fra(X)) syndrome, the most common cause of familial mental retardation, is an X-linked disorder which co-segregates with a fragile site at Xq27.3 (Lubs, 1969; Sutherland and Hecht, 1985). Population surveys in Sweden and England (Gustavson et al., 1986; Webb et al., 1986) have demonstrated that the prevalence of affected males is approximately 1/1250, whereas that of affected females is 1/2000. Sherman et al. (1984, 1985) estimated a penetrance of the mutation in males and females of 79% and 35%, respectively, which led to the prediction that the fra(X) gene frequency would be 1/850.

Since the reproductive fitness of affected males and females is reduced, the fra(X) gene should gradually be lost from the gene pool. In order to compensate for this loss, a high mutation rate has been suggested (Froster-Iskenius et al., 1984; Sherman et al., 1984, 1985; Brown, 1990). When assuming equal mutation rates in egg and sperm, approximately 37% of the affected males are expected to be the result of a new mutation (Sherman et al., 1988).

In order to obtain a more accurate estimate of the proportion of sporadic

cases (i.e. new mutations), we performed genealogic, DNA linkage and cytogenetic studies in conjunction with direct DNA analyses of the CGG repeat in the relatives of 84 fra(X) probands.

MATERIALS AND METHODS

Family Studies

In this study all available ancestors of 80 fra(X) male probands and 4 female probands were included. Diagnosis of the fra(X) syndrome in the probands was based on cytogenetic expression of the fragile site at Xq27.3. Once diagnosed, the subsequent standard procedure was to contact relatives of every proband and visit them at home to collect complete anamnestic information. Based on this information more distant relatives were contacted. In order to trace common ancestors, genealogic data up to the middle of the eighteenth century were collected from local archives.

Cytogenetic Analysis

Blood samples from probands and relatives were cultured in medium 199 (which is low in thymidine and folic acid), supplemented with 5% fetal calf serum. Hundred metaphases of each individual were examined for the presence of a fra(X) chromosome after solid Giemsa staining. Putative fra(X) chromosomes were destained and GTG-banded for confirmation.

DNA Analysis

Polymorphic restriction enzyme site and CA repeat typing for loci closely linked to the fragile site were performed in the genetic workup of 38 families in which the fra(X) syndrome is segregating (Oostra et al., 1990; van Oost et al., 1991, 1992; Riggens et al., 1992). Direct detection of the fra(X) mutation was performed by Southern blot analysis of PstI-digested DNA of 25 fra(X)-positive probands, their mothers and 14 grandparents. Blots were hybridized with pAO365, a 600 bp XhoI-PstI fragment derived just 3' from the CGG repeat (Verkerk et al., 1991). The PstI fragments sizes were classified as follows (van Oost et al., 1992): (i) uniform fragment of normal length and normal intensity (1.0 kbp fragment), (ii) uniform fragment of increased length and a single dosis intensity. (iii) multiple recognizable bands of reduced

intensity identified by the fragment size from which the smear developed upwards, (iiii) complete absence of a hybridization signal with an apparently normal amount of DNA present in the lane.

RESULTS

Anamnestic Analysis of Fra(X) Families

In order to obtain an accurate estimate of the proportion of sporadic cases, all 84 fra(X) probands and their relatives known to us were included. From anamnestic information it became clear that 41 probands had at least one affected sib as well as a more distant affected relative. In 16 probands with mentally normal sibs, the fra(X) syndrome was manifest in one or more distant relatives. Likewise, 19 probands had only one or more affected sibs in the absence of more distant affected relatives. In 8 of the remaining proband families, the family history was unremarkable. Anamnestic information of 3 proband families of this latter group was hard to obtain, as the parents had left the country or were not cooperative.

Genealogical Analysis of Fra(X) Families

Genealogical data were used to find relationships between the probands, and to trace back the mutation. In a total of 11 probands consanguinity could be demonstrated. The common ancestors of 5 probands were traced back to the early 18th century. Two probands were related up to 6 generations ago and for 2 pairs of probands common ancestors were found after ascending 4-5 generations.

Amplification of a CGG Repeat Sequence was Found in all Presumed Carriers

Direct detection of the fra(X) gene was performed via the analysis of the CGG repeat length in 25 affected male probands, their mothers and available grandparents (Table I).

All tested probands, including the 5 isolated fra(X) patients and their mothers as well as all 14 tested grandparents could be identified as gene carrier as they demonstrated the typical size increase and/or dispersion of the CGG

repeat containing PstI fragment. Four of the 7 mothers showed a minor dispersion (defined smear) of the CGG repeat. The fragment size from which the dispersion developed upwards was 1.3-1.6 kb. In contrast to these mentally normal carrier mothers, a full mutation was found in three mentally impaired mothers. Two of the latter group showed a dispersion of the CGG repeat which developed upwards from 1.7 kb and 2.0 kb, respectively, and 1

TABLE I. Distribution of CGG Repeat Length for Fra(X) Male Probands, Their Mothers and Grandparents.

	CGG Repeat Length in Kb (PstI fragment)				
	Normal[a] fragment	Increased[b] fragment	Defined[c] smear	Absence[d] signal	Total
Proband	0	0	18	7	25
Mother	0	18	6	1	25
Grandmother	0	6	1	0	7
Grandfather	0	7	0	0	7
Total	0	31	25	8	64

a: Uniform fragment of normal length and normal intensity (1.0 kbp).
b: Uniform fragment of increased length and single-dose intensity, ranging in size from 1.2-1.6 kbp.
c: Multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards. The fragment size from which the smear developed was 1.4-4.7 kbp.
d: Complete absence of hybridization signal with an apparently normal amount of DNA present in the lane.

mother showed complete absence of hybridization signal. All the affected probands (n=25) carried the full mutation, since a dispersion with an increase in size of 700 to 3700 bp or complete absence of hybridization signal was observed.

Thus, carriership of the fra(X) mutation could readily be detected directly in all available mothers and ancestors of the probands and in many cases also indirectly by using anamnestic information (Table II). In those pedigrees (n=49) in which we were able to trace back the mutation to the grandfather or grandmother of the proband, grandpaternal inheritance of the fra(X) gene could be demonstrated for 25 cases.

TABLE II. Number of Generations in Which the Fra(X) Mutation was Found in Different Proband Families.

Number of generations*	Number of probands
5	10
4	16
3	19
2	20
1a	5
Total	70

* Probands were accounted as generation 1

a For these families carriership of the mother was only proven by CGG repeat analysis.

Expansion of the CGG Repeat Through Generations is not Obligatory

We studied the change in mutation patterns after passage through a Normal Transmitting Male (NTM) and their carrier daughters (Table III). As a general rule, the premutation is transmitted by a NTM to his daughter and changed to a full mutation during oogenesis. However, daughters of a NTM do not show in every instance an increased fragment length similar to that found in their carrier father. Small variations of the fragment, both increased (50-300 bp) and decreased (50-150 bp) in size, were encountered. However, we did not observe any full blown mutation in women carrying the paternal fra(X) mutation. In contrast, a full mutation was found in all but three mutation-bearing grandchildren of NTM's (n=29). Nineteen of the latter group showed multiple recognizable bands of reduced intensity, whereas the

TABLE III. Inheritance of PstI Fragment Length (CGG repeat)

Family	PstI Fragment Length					
	Grandfather	Daughters	Carrier grandsons		Carrier granddaughters	
			Affected	Normal	Affected	Normal
I	0.2	0.4	S 2.0	-	-	-
		0.5	S ?			
		S 0.5				
II	0.4	0.3 (2)	S 4.0 (2)	-	-	-
III	0.2	0.3 (6)	S 5.5 (2)	-	S 1.4	0.2 (2)
		0.5	S 8.0		S 1.5	
		S 0.5	S ? (3)		S ? (5)	
IV	0.35	S 0.4	S 2.0	-	-	-
		S 0.3 (2)				
V	0.5	0.4 (2)	S 1.5	-	-	-
VI	0.4	0.25	-	-	S 1.2	-
VII	0.4	0.35	S 3.8	-	S 2.0	-
		0.4	S 2.0		S 3.8	
VIII	0.4	0.4 (2)	-	-	-	0.4
IX	0.3	S 0.3	S 2.5	-	S 2.4	-
X	0.3	0.3	S ?	-	-	-
		0.5	S 1.8			
XI	0.3	0.3	S 1.8	-	-	-

S : Multiple recognizable bands of reduced intensity as identified by an increased fragment size.

S ? : Complete absence of hybridization signal with an apparently normal amount of DNA present in the lane.

( ) : Number of individuals.

- : Not present.

remaining subjects (n=10) exhibited complete absence of a hybridization signal. In 3 mentally normal granddaughters, the grandpaternal premutation appeared to be unchanged. The CGG repeat analysis was in all cases consistent with the probabilistic diagnosis based on linked DNA markers (van Oost et al., 1992). In Fig. 1 the maternal transmission of the fra(X) mutation, over 3 generations is illustrated.

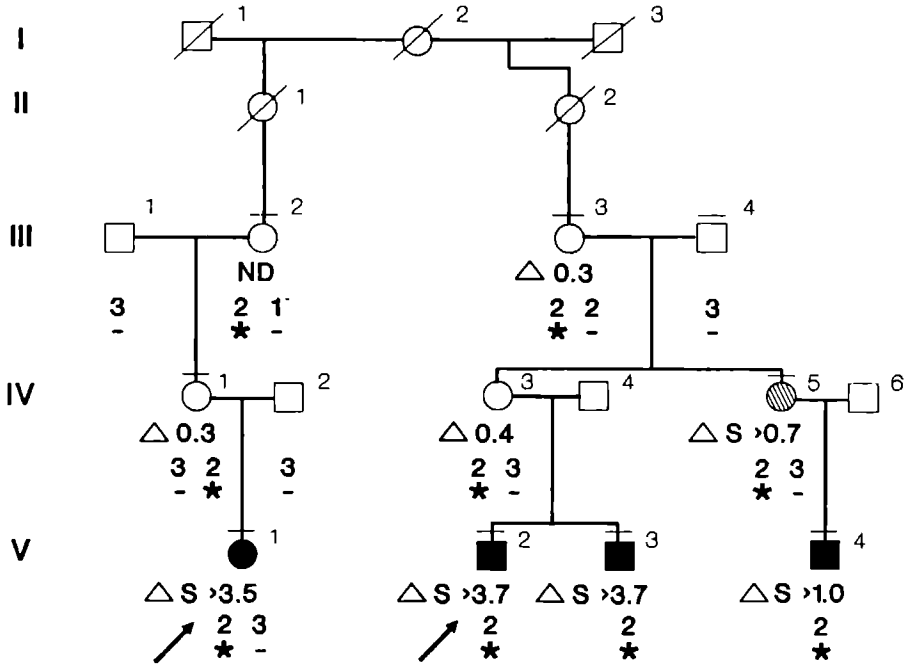

Fig.1. Pedigree of a fra(X) family. The fra(X) mutation is segregating over 4 generations through the maternal line. The * denotes the fra(X) disease locus. The numbers 1,2,3 represents the alleles of the DXS548 locus seen in this family. The fra(X) gene co-segregates with allele "2". △ refers to the increase in size (in kilo base pairs) of the CGG repeat, after PstI digestion. Bar over symbol denotes a cytogenetically negative test, hatched symbol denotes fra(X) positive and mentally normal, solid symbols denote mentally retarded subjects with fra(X) expression. Arrows indicate propositi. ND: CGG repeat analysis not done.

The premutation was introduced into the family by a phenotypically normal female (I-2), who transmitted the mutation to her mentally normal daughters (II-1 and II-2). The available relatives were typed by cytogenetic and DNA-linkage analyses, using the polymorphic DNA marker DXS548 (2), and by direct DNA diagnosis after PstI cleavage. The fra(X) gene in this family co-segregated with allele 2 of the DXS548 locus. Both granddaughters were fra(X) chromosome negative, and Southern blot analysis of Subject III-3 showed an increase in PstI fragment length of 300 bp. The great-granddaughters (IV-1, IV-3, and IV-5) also did not express any clinical symptom of the mutation. However, while 2 of these great-granddaughters were cytogenetically negative and carried a defined increased fragment in the normal transmitting range, the other great-granddaughter (IV-5) was cytogenetically positive (7%) and the CGG repeat was dispersed. In the fifth generation, each individual had a strong increase in fragment size ranging from 1000 to 3700 bp.

Cytogenetic Analysis is Inadequate for Carrier Detection

A total of 37 mothers of the male probands was tested cytogenetically and 7 demonstrated the fra(X) chromosome in 0.5% to 20% of the cells. Three of the 7 mothers appeared to be mentally impaired. The father of one female proband was also cytogenetically tested but was found to be negative. Only 2 of the 23 cytogenetically tested grandmothers of the probands were fra(X) positive, 1.5% and 7% respectively, and none of the 13 cytogenetically tested grandfathers, which indicates that cytogenetic analysis is inadequate for carrier detection.

DISCUSSION

In this study we have shown that in all our informative fra(X) families (n=81) the mutation is familial. This result is based on anamnestic information, supported by cytogenetic and DNA-linkage analyses as well as direct detection of the CGG repeat. In addition, extensive genealogical studies, which we performed in order to find common ancestors of the probands, indicate that the fra(X) mutation might find its origin even more than 270 years ago. Analysis of linkage disequilibrium with the closely linked DXS548 locus in these large families lends further support to this notion (Smits et al., in preparation). Jacobs et al. (1986) reported a dearth of isolated cases of affected males and females in 270 analysed pedigrees. In

addition, results of direct DNA diagnosis in 63 fra(X) families, published recently by Rousseau et al. (1991), showed a lack of evidence for new mutations and the authors concluded that all mothers of affected males and females were carriers of either a premutation or a full mutation. Sherman et al. (1988) reported that about a quarter of all fra(X) probands resulted from new mutations, i.e. were sporadic cases. Even if one assumes that the mutation might develop through several meioses, this seems to be an overestimate because, up to now, we failed to find a new mutation in all tested parents of persons with a premutation.

Several hypotheses have been proposed to explain the development of the fra(X) syndrome (Pembrey et al., 1985; Nussbaum et al., 1986; Laird et al., 1987). The common denominator of these hypotheses is a two-step process. The first step implies the transmission of the premutation to a female, which then has to be passed through oogenesis. In this oogenetic step, the premutation is converted to a full mutation. Our data from families with a male transmitter appear to be consistent with this two- step model, at least as far as most of the carrier grandchildren is concerned. Analysis of families in which the fra(X) syndrome is segregating has shown general patterns of size variation for the CGG repeat unit. NTM's and their daughters show only a small increase of the CGG repeat, while fra(X)-positive individuals in the next generation exhibit much larger fragments that may differ among siblings. These results are in accordance with data, recently published by Oberlé et al. (1991). Thus increase of the CGG repeat length is not obligatory by passage of the premutation through a male and his daughter. The premutation may still be present in grandchildren with grandmaternal transmission of the fra(X) mutation (see Fig.1). These observations confirm results published by Holmgren et al. (1988) concerning large fra(X) families in which the mutation resulted in clinical manifestation of the fra(X) syndrome only after several generations. It can be anticipated that non manifesting carriers with the maternal as well as with the paternal inherited mutation contributes to a large extent to the fra(X) gene pool. This assumption is strengthened by the observation that half of our probands inherited the mutation from their mentally normal grandfathers.

Vogel (1984) reported that an increased fertility of mentally borderline females might contribute to the high incidence of the fra(X) mutation. In our analysis we found no evidence for such an increased fertility among affected female carriers with children (Wilcoxon, $P > 0.45$). On the contrary, the highest number of children was born to carrier females of normal intelligence. However, the number of children of this latter group has as yet

not been compared to a control group.

In conclusion, we did not find any evidence for the presence of sporadic cases, which indicates that the new mutation rate must be negligible, or more likely that the mutation originates by a gradual multigenerational process.

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CHAPTER 3

THE FRAGILE X SYNDROME: NO EVIDENCE FOR ANY RECENT MUTATIONS

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SUMMARY

Fragile X (fra(X)) syndrome, the most common form of familial mental retardation, is caused by heritable unstable DNA composed of CGG repeats. As reproductive fitness of fra(X) patients is severely compromised, a high mutation rate has been proposed to explain the high prevalence. However, we have been unable to show any new mutation for 84 probands referred to us to date. We show here the same fra(X) gene in five fra(X) probands with common ancestors married in 1747. The lack of new fra(X) mutations implies that there must be many more fra(X) gene carriers in the population than previously realized. As it is now possible to detect asymptomatic fra(X) gene carriers by DNA analysis, extended family studies for any new proband are recommended. A family illustrating the importance of fra(X) carriership determination is reported.

INTRODUCTION

The fra(X) syndrome is the most common form of familial mental retardation. Owing to the severity of the disorder, reproductive fitness is greatly diminished in nearly all affected males as well as in a significant proportion of female patients. Therefore, a high mutation rate has been invoked to account for its high prevalence. However, as noted by Jacobs et al., (1986), there appears to be a dearth of new mutations. During the follow up of our 84 index patients, even after going back many generations, we have failed to detect any new mutations (Smits et al., 1992). Similarly, in two other extensive surveys, no new mutations could be found (Rousseau et al., 1991; Yu et al., 1992). An alternative explanation for the lack of new mutations might be recurrent new mutations in the same pedigree. We could discount this latter possibility by showing linkage disequilibrium in a large fra(X) pedigree between the fra(X) gene and a closely linked polymorphic DNA marker. Thus, the fra(X) gene may be transmitted for many generations before becoming manifest. The realization that new fra(X) mutations are very rare has far reaching implications for the counselling of (distant) relatives of fra(X) probands.

MATERIALS AND METHODS

Fra(X) probands were ascertained as described previously (Smits et al., 1992). Genealogical studies were based on municipal documents and

information from the families. DNA extracted from patients' blood samples was analyzed for restriction fragment length polymorphisms, the CA dinucleotide repeat polymorphism at the *DXS548* locus, and for the CGG repeat in the FMR-1 gene (Smits et al., 1992; Smits et al., 1992; Verkerk et al., 1991). The length of the CGG repeat in the normal range was also analyzed by PCR (Fu et al., 1991).

RESULTS AND DISCUSSION

Segregation Analysis in a Large Fra(X) Family

Five fra(X) probands with common ancestors in the early 18th century were ascertained in two different clinical genetics centers in The Netherlands. In all families there were two or more patients displaying the Martin-Bell phenotype and the chromosomal fragile site at Xq27.3. In two probands the fra(X) mutation was inherited from the maternal grandmother and in three probands the maternal grandfather had passed on the fra(X) mutation (fig.1). The highly polymorphic *DXS548* locus is located 140.000 bp proximal to the fra(X) gene locus on the X chromosome (Riggens et al., 1992). *DXS548* can be characterized by PCR analysis of the variable number of CA dinucleotide repeats at this locus. In agreement with our previous studies in a cohort of normal subjects and fra(X) patients from the United States (Riggens et al., 1992), there is no apparent linkage disequilibrium between the *DXS548* marker and the fra(X) locus (see fig.1 for a subset of unrelated fra(X) patients from The Netherlands). However, when the five related probands were tested all showed exactly the same allele of 202 bp (fig.1). As this allele has a frequency of 15% in the population, the chances are less than 1 in 2000 that this result is fortuitous. It is obvious from the pedigree that the fra(X) gene can apparently pass through a number of male and female meioses before becoming manifest. The fra(X) gene has been found to be a segment of unstable DNA (Oberlé et al., 1991; Yu et al., 1991) caused by the expansion of a CGG trinucleotide repeat sequence in the 5' end of the FMR-1 gene (Verkerk et al., 1991). Normal subjects have between six and 46 copies of the repeat, while asymptomatic gene carriers may have 52 to over 200 copies of this repeat (Fu et al., 1991). Fra(X) patients are mosaics in which the repeat might be expanded up to thousands of repeats. Male transmission of the gene is not accompanied by large changes in repeat size, but in female meioses the repeat usually expands. However, this is not obligatory (Rousseau et al., 1991; Yu et al., 1992). It has been hypothesized by Sutherland et al., (1991) and shown by Fu et al., (1991) that it is this plasticity of the fra(X)

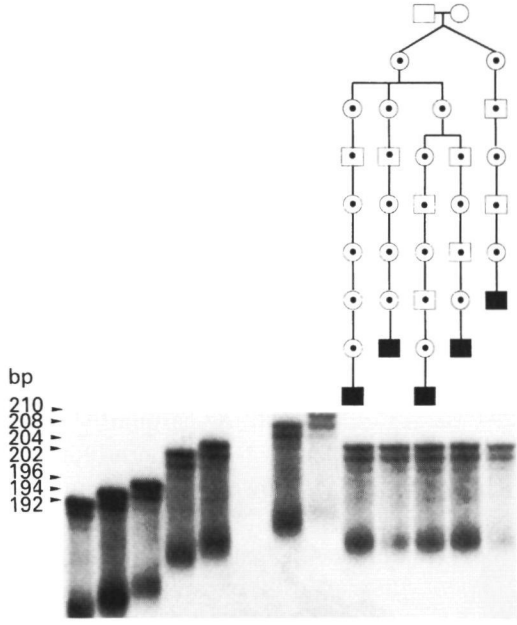

Fig. 1. The polymorphic marker *DXS548* is in linkage disequilibrium with the fra(X) gene in an extended pedigree. Analysis of the CA repeat polymorphism at the *DXS548* locus in seven unrelated fra(X) patients (lanes 1-7) and in five fra(X) probands with common ancestors married in 1747 (lanes 9-12). With specific primers flanking the polymorphic CA repeat, a DNA fragment of 192 to 210 bp was synthesised by the polymerase chain reaction (Fu et al., 1991). The fragments were resolved on a 6% polyacrylamide gel. By labelling one of the primers with^{31} P the fragments could be visualized by autoradiography.

mutation that explains its variable clinical expression and its unusual genetics. Morton et al., (1992) have developed a model in which it is postulated that the expansion of the CGG repeat arises only on X chromosomes which have a larger than normal, but stable, CGG repeat. These alleles may be quite common and only when they increase further in length can the fra(X) syndrome develop in the next generations via female meioses. This model

might also explain the inheritance seen in our pedigree in that the fra(X) has been transmitted many times without significant enough expansion to impede reproduction. Recently, Richards et al., (1992) noted remarkable linkage disequilibrium between the fra(X) locus and two very closely linked CA repeats. The family presented here provides the basis for what these investigators observed in mostly Caucasian populations in the United States and Australia where only a few generations were traced.

DNA Analysis in a Family with an Apparently Isolated Fra(X) Case

The apparent lack of new mutations brings into question whether many more gene carriers might be identified through repeat analysis in any family. That this is likely to be the case is exemplified by our recent analysis of a family with a single patient (fig.2). Subject 1, a cousin of the patient (subject 11), wanted to know her carrier status before becoming pregnant. Linkage analysis with the closely linked flanking DNA markers indicated that the proband had inherited the grandpaternal X chromosome (fig.2, panel A). Direct analysis of the CGG repeat length by Southern blotting (fig.2, panel B) and PCR analysis (fig.2, panel C) showed in all daughters in the second generation an increased fragment of 200 bp, characteristic of the premutation in the fra(X) syndrome. This indicated that the grandfather must have been a 'normal transmitting male'. In the affected grandson (subject 11) a greatly expanded repeat of about 3000 bp in length was seen, while in his phenotypically normal cousin (subject 2), who had also inherited the grandpaternal X chromosome, a less pronounced expansion of the CGG repeat of 500 bp was seen. Of the females in this family, only the grandmother and the consultant did not carry the fra(X) gene.

CONCLUSION

The origin of the fra(X) mutation in the families described here as well as in virtually all other fra(X) families remains elusive. Neither has the conversion of a normal allele to a non-phenotypic premutation allele or to a full mutation been reported. However, the observation that the fra(X) chromosomes of different fra(X) families carry different alleles at the *DXS548* locus makes it unlikely that new mutations do not occur. Indeed, there is no a priori argument in favor of the absence of new mutations. It may take an as yet undetermined number of generations before the fra(X) mutation surfaces. In view of this latency of the fra(X) mutation, quite distant relatives of any

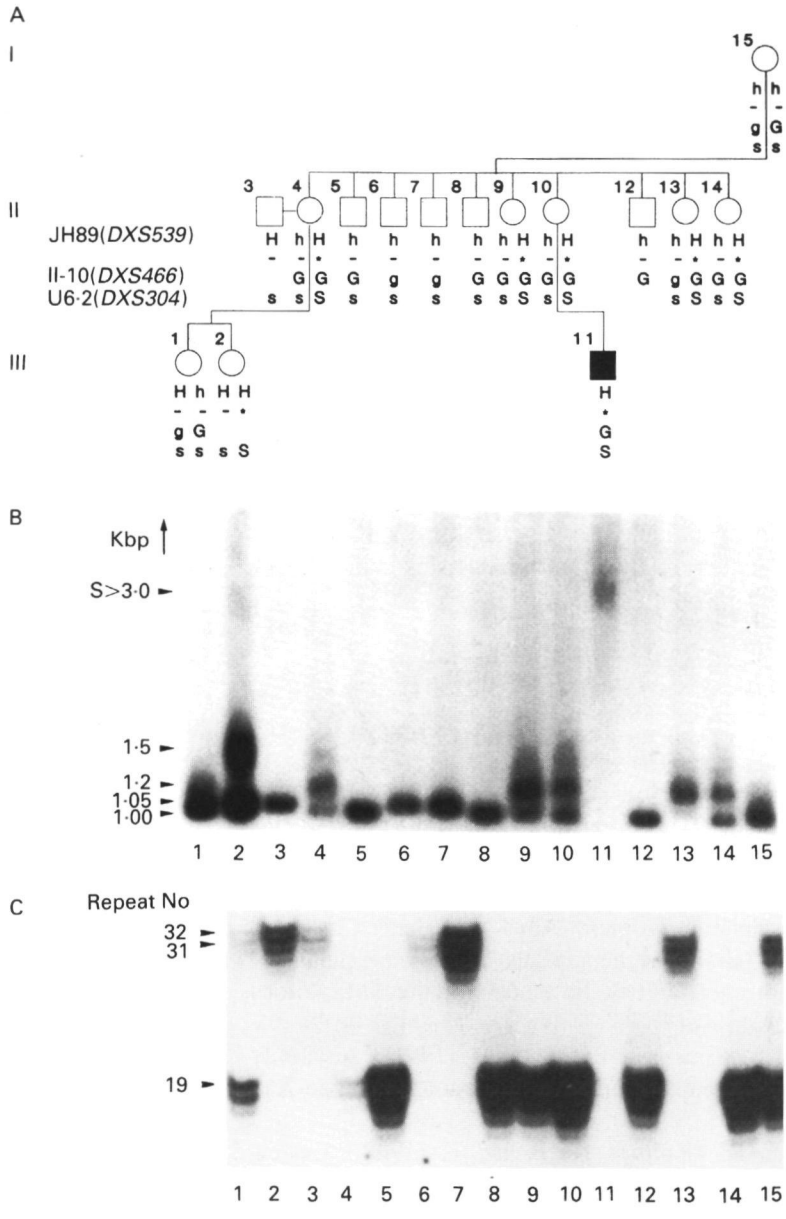

Fig. 2. DNA linkage and direct mutation analysis in a family with a single fra(X) patient. Analysis of the polymorphic DNA markers *DXS539* (Hh),

DXS466 (Gg), and *DXS304* (Ss) (upper panel A), and direct analysis of the CGG repeat by Southern blotting of PstI digests of the patient's genomic DNA (middle panel B), and by PCR with primers flanking the CGG repeat (lower panel C). In the pedigree the at risk allele is indicated by an asterisk. For subject 13, the Southern blot is not unequivocal as the signal of the normal X chromosome is not clearly distinguishable. This can be explained by the fact that this subject inherited the maternal X chromosome with the larger, but still normal, CGG repeat from her mother, the same allele as her two brothers (subjects 6 and 8). Owing to the inefficiency of the PCR for longer CGG inserts, the product of the expanded alleles in the gene carriers could not be shown. Only the consultant and her grandmother show heterozygosity for the normal sized alleles of the CGG repeat, confirming their non-carriership.

fra(X) proband may be at risk for fra(X). Therefore, our data suggest that carrier detection by DNA analysis is indicated throughout extended families identified through new probands, and that population based screening may be warranted.

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CHAPTER 4

PENETRANCE OF FRA(X) GENE: INFLUENCE OF GRANDPARENTAL ORIGIN OF THE GENE, MENTAL STATUS OF THE CARRIER MOTHER, AND PRESENCE OF A NORMAL TRANSMITTING MALE

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SUMMARY

Previous studies have indicated that the fragile X (fra(X)) gene does not show full penetrance (mental impairment) in carrier females or "carrier" males. The phenomenon of non-expressing carrier males distinguishes the fra(X) syndrome from all other known X-linked disorders. Moreover, penetrance of the fra(X) gene apparently does not show random distribution within fra(X) families, but seems to be reduced in sibs of normal transmitting males (NTM's). The availability of many large multi-generation fra(X) families, studied by cytogenetic and DNA analyses, enabled us to refine the estimates of the penetrance. From our data we conclude that the penetrance in daughters of carrier females is determined by the mental status of the mother. In sons of carrier females, the observed penetrance appears to be influenced by the grandparental origin of the gene as well as by the mental status of the mother. However, in contrast with the average penetrance, we observed a strongly reduced penetrance of the fra(X) gene in brothers (14%) and sisters (21%) of NTM's. These findings have profound implications for genetic counseling.

INTRODUCTION

Since preliminary investigations in our fra(X) families pointed to a much higher penetrance (mental impairment) in females than reported thus far, we decided to study systematically the penetrance of the fra(X) gene. We were able to perform extensive family studies in 77 multi-generation pedigrees. Based on these data, including genealogic and cytogenetic results supported by DNA linkage studies and direct molecular analysis of the fra(X) mutation (Oostra et al., 1990; van Oost et al., 1991; 1992; Smits et al., 1992a), we present a refinement of the estimated fra(X) penetrance in the offspring of carrier females. The large number of multi-generation families allowed us to calculate different penetrance figures with regard to the mental status and the parental origin of the fra(X) gene in the carrier mother. In addition, the penetrance in brothers and sisters of normal transmitting males (NTM's) could be established. The results have direct implications for genetic counseling of fra(X) families.

MATERIALS AND METHODS

Family Studies

The index patients were referred to us for cytogenetic analysis of the fra(X) chromosome by clinical residents in public hospitals and institutions for the mentally retarded. The standard procedure was to contact the family after the fra(X) syndrome had been diagnosed in the proband. Relatives were all visited at home, where a detailed family history was taken and the intellectual level of the relatives was assessed. All at-risk relatives who consented were tested cytogenetically and, if necessary, also at the DNA level. Obligate carrier mothers were classified according to their mental status. Two levels could be differentiated, normal and borderline. Normal mental status implied that no obvious signs of mental disability were present. "Borderline females" were mildly affected as they had significant learning disabilities, but were self-supporting in society. The mental status of children of obligate carrier mothers was obtained anamnestically. All children who were learning-disabled according to school criteria and received special education were classified as mentally impaired.

Cytogenetic Analysis

Blood samples of all individuals were cultured for 96 hours in medium 199 supplemented with 5% fetal calf serum. One hundred metaphases of each individual were examined for the presence of a fra(X) chromosome after solid Giemsa staining. Putative fra(X) chromosomes were destained and GTG-banded for confirmation.

DNA Analysis

DNA-linkage analysis and direct detection of the CGG repeat length were performed in relatives to diagnose carriers and to trace back the origin of the fra(X) gene. The analyses were carried out as described in the accompanying paper (van Oost et al., 1992).

Six polymorphic DNA probes used, St14 (DXS52) and U6.2 (DXS304) as distal markers, and RN1 (DXS369), JH89 (DXS539), VK23B (DXS297), 55E (DXS105) as proximal markers. In addition, we applied a highly polymorphic dinucleotide repeat, RS46 (DXS548), which is tightly linked to the fra(X)

syndrome locus (Riggins et al., 1992).

Direct detection of the CGG repeat length was assessed via Southern blot analysis of PstI-digested total genomic DNA. The blots were hybridized with a 600 bp XhoI-PstI fragment just 3' from the CGG repeat sequence (Verkerk et al., 1991). The PstI fragment sizes were classified as follows: (i) uniform fragment of normal length and normal intensity (1.0 kbp fragment), (ii) uniform fragment of increased length and a single dosis intensity, (iii) multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards, (iiii) complete absence of a hybridization signal with an apparently normal amount of DNA present in the lanes.

Segregation Analysis

Direct estimate of the penetrances was performed by taking twice the relative frequency of affected sons and daughters. The numbers of observed mentally impaired sons and daughters were evaluated with all possible penetrances (0.0-1.0) using a Chi-square test. A 95% confidence limit gives an interval estimate for the mean of the penetrance.

RESULTS

Since the time that fra(X) studies in our laboratory were initiated, we have detected the fra(X) gene in 77 multi-generation families. In these families we were able to identify more than 200 carrier mothers. After ascertainment correction, by omitting index families (proband, sibs and parents) completely, a total of 100 nuclear families, including 347 children with defined phenotypes, were subjected to penetrance analysis. Estimation of the penetrance in sibs of NTM's was performed in 21 families in which a NTM was identified. Ascertainment correction was applied by excluding the index NTM's, leaving 101 sibs with defined phenotypes.

Penetrance in Sons and Daughters of Obligate Carrier Females

As there were 80 mentally impaired sons out of a total of 188, a penetrance of 85% (95% confidence limits: 72-98%) could be deduced for carrier males (Fig.1a).

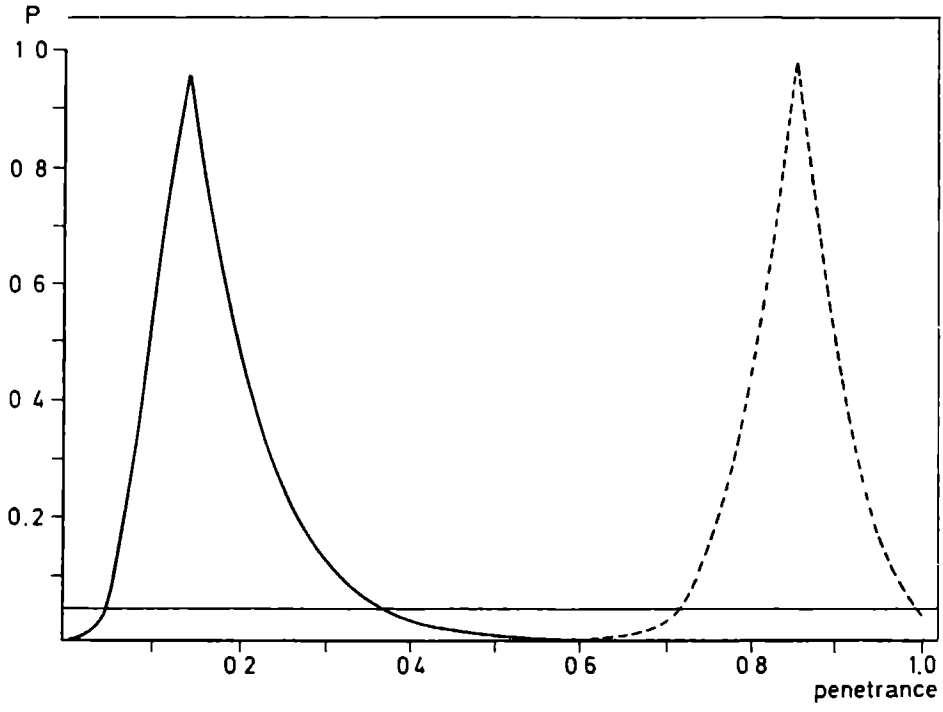

Fig. 1a. Penetrance of the fra(X) gene in males.
------ Sons of carrier females.
—— Brothers of normal transmitting males.
Y axis: P value of Chi-square test. The top of the curve corresponds to the estimated penetrance, whereas a horizontal line can be drawn to indicate the 95% confidence limits for the penetrance.

For carrier females, fifty-one mentally impaired daughters out of a total of 159 resulted in a penetrance of 64% (95% confidence limits: 52-79%) (Fig.1b).

In Table I, the penetrance in sons and daughters of mentally normal and borderline carrier mothers in relation to the grandparental origin of the fra(X) gene is presented.

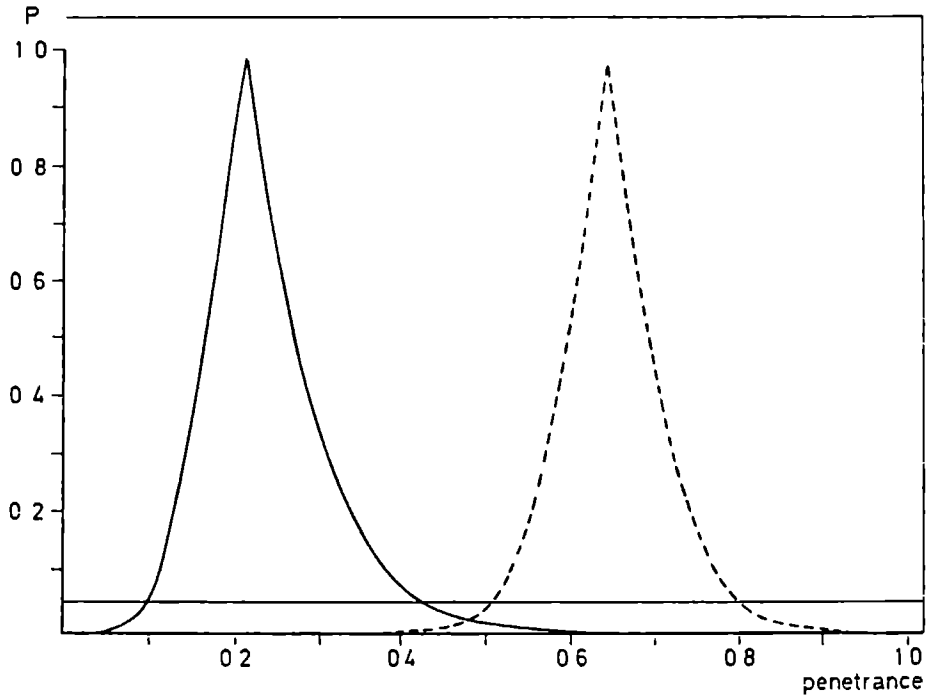

Fig. 1b. Penetrance of the fra(X) gene in females.
------ daughters of carrier females.
—— sisters of normal transmitting males.
For further details see legend to Fig.1a.

The penetrance in sons appears to be influenced by the grandparental origin of the fra(X) gene. We observed full penetrance (95% confidence limits: 78-100%) in sons who carry the grandpaternal fra(X) gene, whereas 76% (confidence limits: 49-100%) of the sons who carry the grandmaternal fra(X) gene were affected. The highest penetrance in this latter group was seen in sons of mentally borderline mothers, namely 86% (95% confidence limits: 44-100%) versus 69% (95% confidence limits: 36-100%) in sons of mentally normal mothers. The penetrance in daughters of carrier females is not related to the grandparental origin of the fra(X) gene, as daughters of mentally normal mothers carrying the grandpaternal or grandmaternal fra(X) gene

TABLE I: Penetrance in Children of Mentally Normal and Borderline Female Carriers Related to the Origin of the Fra(X) Gene.

Origin fra(X)	Mother mental status	N	Sons penetrance %	N	Daughters penetrance %	N
Grandpaternal	Normal	41	100	70	56	57
	Borderline	1	0	1	100	2
	Unknown	0	---	---	---	---
Grandmaternal	Normal	18	69	29	57	28
	Borderline	13	86	21	100	19
	Unknown	4	91	11	50	4
Unknown		23	71	56	61	49
Overall		100	85	188	64	159

showed penetrances in the same range, 56% and 57%, respectively. Full penetrance (95% confidence limits: 58-100%) was found in daughters of borderline intelligent mothers. These borderline intelligent mothers received in all but one the maternal fra(X) mutation.

Penetrance in Brothers of Normal Transmitting Males

For several years, identification of NTM's could be accomplished only by pedigree analysis and DNA-linkage studies. Recently, NTM's and non-expressing carrier males of the fra(X) mutation can easily be recognized at the molecular level by an increased CGG fragment length. Figure 2 shows some examples of the length variation of the PstI restriction fragment encompassing the CGG repeat. A fra(X) family over 3 generations is shown, including a NTM and his mentally normal brother. The grandfather of the affected children had already been proven to be a NTM by DNA-linkage analysis with the tightly linked flanking markers VK23B (DXS297) and

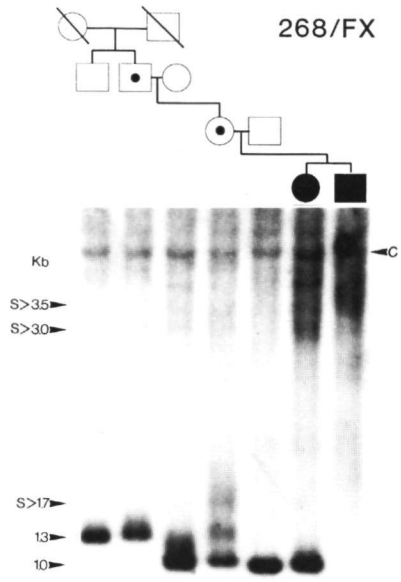

Fig. 2. Southern blot analysis of the CGG repeat length in a three generation fra(X) family (268/FX). DNA's were cleaved with PstI and blots were hybridized with the 600 bp XhoI-PstI fragment just 3' of the CGG-repeat sequence. In the mentally normal brother of a normal transmitting male (lane 1) the normal 1.0 kb fragment length is replaced by a fragment of 1.3 kb. The same increased fragment length is seen in lane 2, which contains DNA from the normal transmitting male. Lane 4 (an obligatory carrier female with the paternally inherited fra(X) gene) shows a fragment of normal length, a fragment of increased length at 1.3 kb and a dispersed segment >1.7 kb. Lane 6 displays a normal fragment of 1.0 kb as well as a dispersed segment of >3.0 kb of the affected daughter. Lane 7 contains DNA from the affected son who shows a dispersed segment of >3.5 kb. Lane 3 and 5 show the normal fragments of the two spouses.

pU6.2 (DXS304). A fragment of increased length which replaces the normal one can be observed in the NTM (lane 2) as well as in his mentally normal brother (lane 1).

Twenty-one males were identified as NTM's on the basis of extensive pedigree analyses. In 12 cases this could be confirmed by DNA-linkage studies using flanking markers (accuracy >99%). Eleven of the 12 molecularly studied NTM's were also tested by direct analysis of the CGG

repeat, which showed an increase in length ranging from 0.2-0.5 kb (Smits et al., 1992b), so that the diagnoses were confirmed.

The penetrance of the fra(X) gene in brothers of NTM's was analysed in 44 individuals with defined phenotype. In 3 cases, anamnestic information demonstrated mental impairment, which resulted in a penetrance of 14% (95% confidence limits: 4-37%) (Fig.1a). In 2 of the 3 mentally impaired brothers it was impossible to confirm the presence of the fra(X) syndrome because they were already deceased. The third male was indeed mentally impaired and found to be fra(X) positive upon cytogenetic investigation.

Nine phenotypically normal brothers of NTM's also turned out to be NTM's. This result was obtained by pedigree analysis, while in 2 families the diagnosis was confirmed by DNA-linkage analysis using marker RS46 (DXS548), as well as by direct evaluation of the CGG repeat length (Fig.2). In another family 3 NTM's, including the index NTM, were identified. One of them was deceased but could be identified via the use of flanking markers, proximal pVK23 (DXS297) and distal pSt14 (DXS52), in his offspring. The other 2 NTM's were recognized by flanking markers as well as direct detection of an 0.4 kbp increase of the CGG repeat.

Penetrance in Sisters of Normal Transmitting Males

Penetrance of the fra(X) gene in sisters of NTM's was analyzed in 57 cases. Six females showed mental impairment which results in a penetrance of 21% (95% confidence limits: 10%-43%) (Fig.1b). Two of the 6 affected sisters were cytogenetically tested and showed 9% and 2% fra(X) expression, respectively. The female with 2% fragile site expression was also tested for the CGG repeat length and showed an increased fragment which presented as multiple recognizable bands (defined smear). The fragment size from which this smear developed upwards was 1.5 kbp.

Sixteen phenotypically normal sisters appeared to be carrier because the fra(X) syndrome was manifest in their offspring. Another 6 mentally normal sisters were cytogenetically tested and all were negative. However, flanking DNA markers pointed to carriership of the fra(X) gene in 3 of them. This result was confirmed by direct detection of the CGG repeat length, as judged from Southern blots of EcoRI digests. In these 3 carrier sisters of NTM's an increase in CGG repeat length of 0.1 kbp was seen.

DISCUSSION

It is well established that apparently unaffected males are able to transmit the fra(X) gene to their daughters (Martin and Bell, 1943; Webb et al., 1981; Fryns and Van den Berghe, 1982; Gardner et al., 1983; Froster-Iskenius et al., 1984; Pembrey et al.,1984; Brown et al., 1985; 1987; Howard-Peebles and Friedman, 1985; Loesch et al., 1987; Holmgren et al., 1988). Most of these reports do not mention affected brothers and sisters of such NTM's. Assuming that there is no segregation distortion (Sherman et al., 1988), we observed a strongly reduced penetrance (mental impairment) in brothers as well as in sisters of NTM's: 14% and 21%, respectively, against 85% in sons and 64% in daughters of carrier females. (Fig. 1a and 1b). Sherman et al. (1985) noted an even lower penetrance of 10% in sisters of NTM's. However, such an extreme low penetrance might be caused by the POINTER program, as used by Sherman et al. (1985]), which leads to an underestimate of the penetrance in females (de Haan et al., 1992).

The penetrance in daughters of carrier females appears to be considerably higher than the overall penetrance in females reported earlier, 64% versus 35% (Sherman et al., 1985). This discrepancy might be explained by the absence of daughters of NTM's who are always non penetrant, or by different ascertainment corrections (de Haan et al., 1992). Although our estimate of 64% penetrance in females is somewhat higher than the approximately 50% penetrance obtained in a number of other studies (Fishburn et al., 1983; Turner and Jacobs, 1983; Sherman et al., 1988; Navajas and Vianna-Morgante, 1989; Cronister et al., 1991), such a discrepancy might be explained to a large extent by the different criteria of mental impairment. In our study, all females who were learning-disabled according to school criteria were classified as mentally impaired. Loesch and Hay (1988) reported that up to 85% of heterozygotes demonstrated cognitive deficits. We detected such a high penetrance in daughters who received the grandmaternal fra(X) gene via their borderline functioning mother (Table I). An equally high penetrance in daughters of mentally impaired mothers was recently reported by Cronister et al. (1991). In carrier daughters who received the fra(X) gene from their mentally normal mother a much lower penetrance independent of the grandparental origin of the gene was seen.

The penetrance of the fra(X) gene in sons of carrier females is in the same range as the overall penetrance in males reported earlier (Sherman et al., 1985). The mentally borderline mothers as well as the mothers who carry the paternal fra(X) gene contributed to the high penetrance in sons of carrier

females. It is tempting to speculate that NTM's would have received the grandmaternal fra(X) gene via their mentally normal mother. However, we were able to detect the grandpaternal fra(X) gene in 3 non-expressing males who were identified by DNA-linkage analysis in combination with direct detection of the CGG repeat length. Anamnestic information regarding the mental status of mothers of NTM's is difficult to obtain, as most of them had to be traced back at least 4 generations ago. In the 12 cases in which we could obtain reliable anamnestic information, no evidence for the presence of mentally borderline mothers of NTM's was found.

Our results have important implications for genetic counseling as they indicate that clustering of non-expressing fra(X) carrier males and females is a common phenomenon. We found no evidence that the reduced penetrance in mentally normal sibs of NTM's is caused by a segregation distortion of the fra(X) gene, as 25 out of the 28 expected carrier females could be positively identified by either pedigree and/or DNA analyses. This implies that mentally normal sibs of affected males have considerably less risk of carrying the mutated gene, compared to sibs of NTM's. Based on this study, the risk for a brother of an affected male is 13%, while the risk for a brother of a NTM is 46%. The same holds true for mentally normal sisters of affected brothers who have 26% risk to carry the fra(X) gene, while sisters of NTM's have a risk of 44%. Therefore, an early identification of NTM's is of great importance for timely carrier detection, particularly in branches of pedigrees in which no clinical or cytogenetic expression of the fra(X) syndrome is manifest. This can be achieved readily now the fra(X) gene has been characterized (Bell et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Yu et al., 1991) and NTM's can be identified via CGG trinucleotide repeat analysis.

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CHAPTER 5

PENETRANCE ESTIMATE OF THE FRA(X) GENE USING POINTER VERSUS DIRECT ESTIMATE

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SUMMARY

Direct estimate of the penetrance of the fra(X) gene was compared with the estimate using the Pointer computer program. Direct estimate gave overall penetrances of 85% for male and 64% for female carriers. The estimates calculated by the Pointer program were 82% for males and 38% for females. It is argued that the use of the Pointer program gives incorrect estimates of the penetrance of the fra(X) gene.

INTRODUCTION

The fragile X (fra(X)) syndrome is a familial X-linked form of mental retardation. The syndrome is known to have incomplete penetrance in males as well as in females. Recently, Smits et al. (1992a) reported estimates of the overall penetrance, as defined by mental impairment, for males (85%) and females (64%) in the offspring of obligate female carriers. Especially, the estimate reported for daughters differed significantly from the 35% penetrance in females published by Sherman et al. (1985). This large difference in penetrance has considerable implications for genetic counseling.

The purpose of this study was to investigate this discrepancy. We addressed the question whether this discrepancy arose because of different populations or because of different analysis of the data.

MATERIALS AND METHODS

Direct Estimate of the Penetrance

The direct method of estimating the penetrance, as defined by mental impairment, started with examining 77 pedigrees for obligate fra(X) female carriers. To avoid ascertainment bias the proband families (proband, sibs and parents) were omitted. The offspring of the obligate female carriers were investigated. A total of 100 obligate female carriers with 347 children was incorporated in this part of the study. The direct estimate of the penetrance for males is twice the relative frequency of affected males in the offspring. The same procedure gives direct estimates for females. Persons with significant learning disabilities according to school criteria were classified as affected.

TABLE I: Complex Segregation Analysis with the Pointer Program (77 Pedigrees).

Model	χ^2 + constant	Estimations of the parameters of the model d	t	q	m	Derived penetrance values males	females
1	740.84	0.50	15.94	0.00054	(0.90)	0.81	0.38
2.	740.84	(0.50)	15.99	0.00054	(0.90)	0.81	0.38
3.	742.69	0.51	15.64	0.00054	(0.80)	0.81	0.38
4.	742.69	(0.50)	16.00	0.00054	(0.80)	0.82	0.38

Parameters in parenthesis are fixed
χ^2 + constant: -2 ln(likelihood) to which the Pointer program omits a constant value independent of the model parameters

Estimate of the Penetrance Using the Pointer Program

Complex segregation analysis was performed on the above mentioned 77 pedigrees by the Pointer program developed by Morton et al. (1983). Only families which fulfilled the same criteria as used by Sherman et al. (1985) were incorporated in our study. This resulted in 244 families with 984 children that were used in the Pointer analysis.

In short, the following parameters were used. The selection coefficient, "m", against males is the probability of no transmission of the fra(X) gene for males. The population gene frequency is denoted by "q" and the dominance for females by "d". A higher "d" results in a higher penetrance, d=0 means recessive, d=1 means dominant. Furthermore, the parameter "t" gives a measure for the distance (on a liability scale) between male carriers versus normal males, a higher "t" results in a higher penetrance. This distance is assumed to be the same as the distance between the two homozygous female classes. Other parameters in the model were fixed on the default values. The

ascertainment probability was set on 0.05. See Sherman et al. (1985) for the full description of the parameters of the model used by the Pointer program.

The estimates of the population prevalence of males and females were set on 0.00044 and 0.00041 as used by Sherman et al. (1985). A number of models were fitted. Each model gives estimates of the penetrances in male and female carriers.

RESULTS

Direct Estimate of the Penetrance

As there were 80 mentally impaired male offspring out of 188, a penetrance of 85% could be deduced for male carriers. For females, 51 mentally impaired offspring out of 159 resulted in a penetrance of 64% for female carriers.

Estimate of the Penetrance Using the Pointer Program

Four different models were fitted. The models differed on the selection coefficient, $m=0.80$ and $m=0.90$, respectively, and on the possibility to iterate the dominance parameter. The results of the fitted models are presented in Table I. Fixing the dominance parameter or free iteration gives for both selection coefficients the same estimates. The estimates of the penetrance are almost the same for all 4 models and are similar to the estimates of Sherman et al. (1985).

DISCUSSION

In this study it is demonstrated that estimates of the penetrance by the computer program Pointer are different from those directly deduced. In particular, the 38% penetrance of the fra(X) gene in female carriers obtained by the Pointer program differs dramatically from the 64% penetrance estimated by the direct method. It might be argued that the direct estimate is too high because of insufficient ascertainment correction, in particular by not taking into account the patients or obligate carriers which were used to establish carriership in relatives of the proband families. To test this assumption, we also removed from the data set all offspring which was used

to establish carriership of the mother. The resulting direct penetrance estimate of 61% for daughters of obligate carriers was only fractional lower than without this extra ascertainment bias correction. We may conclude that insufficient ascertainment correction cannot be held responsible for the discrepancy between the results of the Pointer program versus the direct estimate. On the other hand, the low estimate calculated by the Pointer program might have been caused by the inclusion in the data set of normal transmitting male families. Daughters of normal transmitting males do express the gene very rarely (Sherman et al., 1985; Smits et al., 1992a) and inclusion of these females in the data set might have caused the lower penetrance in female offspring as calculated by the Pointer program. However, reanalysing the same dataset without the 29 families of normal transmitting males resulted in the same estimates of the penetrance. Even using the Pointer program to analyse only the families of obligate female carriers did not affect the penetrance. Therefore the conclusion that the Pointer program gives artificially low penetrance values for female carriers seems inescapable.

Inspection of the assumptions on which the Pointer program is based suggests to us that the direct method results in more reliable data than the computer program. In the theory on which the Pointer program is based we found in the derivation of the transition matrices in the case of a sex-linked disease the following assumption (Morton and Yashuda, 1979): "The selection coefficient is taken to be m against the A' gene in males, but is negligible in females." For most sex-linked diseases this is a correct assumption, but in the fra(X) syndrome affected females do not have a negligible selection coefficient. Of the 51 mentally impaired females in our study 32 were adults. Of those 32 adults 13 were not self-supporting (institutionalized). The reduced reproductive fittness of the retarded females is not compensated by a larger number of offspring of the mildly retarded carrier females. In our material, mildly impaired mothers do not have more children on the avarage than normal mothers (Smits et al., 1992b). This gives an estimate of the selection coefficient for females of (13/32) times 64% = 26%. If, as in the fra(X) case, the selection coefficient against females is wrongly chosen to be zero, it follows implicitly that the gene frequency in females (and to a lesser degree also in males) will be overestimated. With the same prevalence (that is the number of affected individuals in the population) this results in an underestimate of the penetrance (number of affected divided by number of carriers). Furthermore, the estimation of the penetrance by the Pointer program strongly depends on the prevalence, for which precise data are lacking.

We conclude that it is incorrect to use the Pointer program for estimating penetrance and gene frequency in the fra(X) syndrome. The same will hold for other sex-linked diseases with positive selection against females.

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CHAPTER 6

PARENTAL ORIGIN OF THE FRA(X) GENE IS A MAJOR DETERMINANT OF THE CYTOGENETIC EXPRESSION AND THE CGG REPEAT LENGTH IN FEMALE CARRIERS

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SUMMARY

The fragile X (fra(X)) syndrome is the most frequent form of inherited mental retardation, and co-segregates with a fragile site at Xq27.3 as well as with insertion of a variable number of trinucleotide repeats in the 5'-end of the FMR-1 gene. As the fra(X) gene is transmitted by females as well as males, we have investigated whether the parental origin of the fra(X) gene has an effect upon the cytogenetic expression and CGG repeat length. An increased fragment length of 0.2 - 0.6 kb appeared to be associated with a very low expression or even complete absence of the fragile site as well as with a normal phenotype, and was seen mostly in cases of paternal inheritance. However, in most female carriers with the maternally inherited fra(X) gene we found dispersed fragments ranging from 1.4 - 6.5 kb or even complete absence of a hybridization signal. Within the group of female carriers with the maternally inherited fra(X) gene we found a statistically significant correlation between the level of the cytogenetic expression and the PstI restriction fragment length encompassing the CGG repeat. These data can be taken as indirect evidence that CGG repeat length and cytogenetic expression are causally related.

INTRODUCTION

In the fragile X (fra(X)) syndrome, which is considered to be the most common familial form of X-linked mental retardation, an unusual mode of inheritance is seen. In contrast to many other severe X-linked diseases, the fra(X) gene can be transmitted by phenotypically normal males and penetrance is appreciable in females (Sherman et al., 1984; 1985; Tommerup, 1989; Smits et al., 1992).

Recently, the fra(X) gene has been molecularly cloned and sequenced in part (Oberlé et al., 1991; Yu et al., 1991; Verkerk et al., 1991). Carriers can be discriminated on the basis of an increase in length and dispersion of a restriction fragment in the 5'-end of the FMR-1 gene (Oberlé et al., 1991; Yu et al., 1991). It is thought that the length variation of this restriction fragment is due to the expansion of a CGG trinucleotide repeat (Kremer et al., 1991). It has been suggested that the extent of the length increase of this segment may be associated with the clinical and cytogenetic expression as well as by the parental origin of the gene (Oberlé et al., 1991). We have reevaluated our extensive fra(X) family material for the CGG repeat length variation. This enabled us to compare female carriers with the paternal fra(X) chromosome

with the group of female carriers with the maternal fra(X) chromosome. Here we confirm that the parental origin of the fra(X) chromosome is a major determinant for the length variation of the CGG repeat in female carriers. We also demonstrate a significant correlation between the CGG repeat length and the cytogenetic expression of the fragile-X site.

MATERIALS AND METHODS

Subjects

From a total of 200 families, we selected 110 obligate female carriers in whom mental status assessment, cytogenetic typing and/or Southern blot analysis was done. It was established by pedigree analyses that 41 females carried the paternal fra(X) gene and 69 females the maternal fra(X) gene. The mental status in the female carriers was classified as normal, borderline or retarded. Borderline was defined as mildly affected (learning disabilities) but self-supporting in society. Retarded females were moderately affected and not self-supporting in society.

Cytogenetic Analysis

Peripheral lymphocytes of all carriers were cultured for 92 hours in medium TC 199, supplemented with 5% fetal calf serum. Chromosome slides were made according to routine procedures. One hundred metaphases of each individual were examined for the presence of a fra(X) chromosome after solid Giemsa staining. Potential fra(X) chromosomes were photographed, destained, and subsequently GTG-banded for evaluation.

DNA Analysis

CGG repeat length was assessed via Southern blot analysis of PstI and EcoRI digested total genomic DNA. The blots were hybridized with a 600 bp XhoI-PstI fragment just 3' from the CGG repeat (Verkerk et al., 1991) as described in detail in the accompanying paper (van Oost et al., 1992). Only those carriers were included in this study in whom a normal size EcoRI and/or PstI fragment with a single dose intensity, representing the normal X-chromosome, was observed. For the abnormal chromosome the PstI and EcoRI fragment sizes were classified as follows: (i) uniform fragment of

normal length and normal intensity (either the 1.0 kbp PstI or the 5.1 kbp EcoRI fragment), (ii) uniform fragment of increased length and a single-dose intensity, (iii) multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards, (iiii) complete absence of hybridization signal with an apparently normal amount of DNA present in the lanes. As the signals derived from the PstI digests allowed a better classification of the hybridization patterns, all within- and inter-group comparisons were based primarily on the PstI digests.

RESULTS

Paternal Transmission

Thirty-seven of 41 females, who inherited the paternal fra(X) gene, showed no fra(X) expression. The other 4 female carriers showed only 1-2% expression of the fragile site. Both cytogenetically negative and cytogenetically positive carriers were mentally normal, and the average ages in both groups of, respectively, 42 and 41 years did not differ significantly. In Figure 1 the length variation in the CGG repeat containing restriction fragments is shown in a fra(X) family over 3 generations. The grandfather of the affected children was identified as normal transmitting male by pedigree analysis (grandchildren of his brother were also affected) and DNA-linkage analysis with the linked markers JH89 (DXS539) and F814 (DXS52) flanking the fragile site (Dreesen et al., unpublished data). In the cytogenetic positive and mentally retarded male patient, a dispersed signal is seen for both the EcoRI and PstI digests (Fig. 1a,b, lane 4). The apparent increase in size, as judged from the best discernible signal, was at least 1500 basepairs for the EcoRI digest and at least 3000 basepairs for the PstI digest. The apparently larger increases in PstI fragment length as compared to EcoRI was a consistent finding in all patients studied (unpublished data). In the PstI digest of DNA from the grandfather an abnormal fragment replacing the normal one is seen (Fig. 1b, lane 1). His daughter shows, besides the normal size band representing the normal maternal X-chromosome, a fragment of increased length slightly smaller in size as the one seen in her father (Fig. 1b, lane 2). For the EcoRI digests qualitatively similar data were obtained but, due to the limited resolution of the agar electrophoresis, the size differences were less pronounced (Fig. 1a, lanes 1 & 2). The Pst I digests of in total 32 female carriers with the paternal fra(X) gene were analysed for the CGG repeat length and 29 out of this group showed a well defined fragment which was increased in length from 0.2 - 0.6 kb (Table I). The average increase in PstI

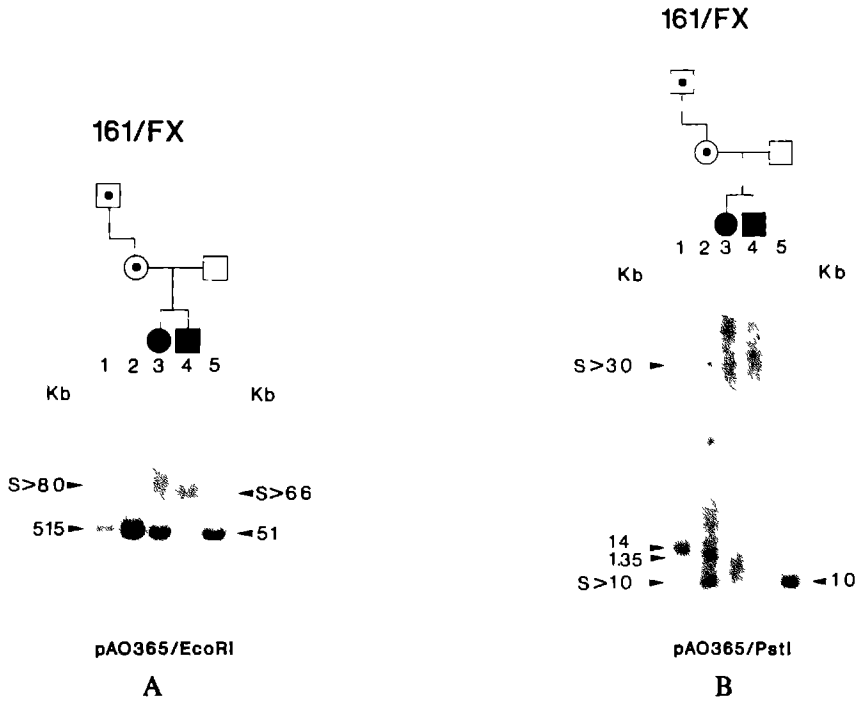

Fig. 1. Southern blot analyses of a three generation fra(X) family (161/FX). Blots were hybridized with the XhoI-PstI fragment just 3' of the CGG repeat sequence. Lane 5 contains DNA from the unaffected spouse. For further details see text. A: EcoRI digest; B: PstI digest.

fragment length of 410 basepairs in the cytogenetic positive group (n=3) was not significantly different from the average increase in PstI fragment length of 380 basepairs seen in the cytogenetic negative group. Three female carriers, of which one had 1% cytogenetic expression, showed a slightly dispersed signal in the same size range.

Maternal Transmission

Female carriers who inherited the maternal fra(X) gene (n=69) were classified according to their mental status and cytogenetic expression (Table II). Only 5 out of a group of 15 mentally normal females demonstrated cytogenetic expression, with a range from 1% to 14%. All borderline and

TABLE I. Distribution of CGG Repeat Length in Cytogenetic Negative and Positive Females Carrying the Paternally Inherited Fra(X) Gene.

	CGG Repeat Length in Kbp (PstI fragment)				
	Normal[a] fragment	Increased[b] fragment	Defined[c] smear	Absence[d] signal	Total
Cytogenetic Negative	0	26	2	0	28
Cytogenetic Positive	0	3	1	0	4
Total	0	29	3	0	32

a: Uniform fragment of normal length and normal intensity (1.0 kbp).
b: Uniform fragment of increased length and single-dose intensity, ranging in size from 1.2-1.6 kbp.
c: Multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards. In this sample the fragment were only slightly dispersed over a range of 1.45-1.50 kbp.
d: Complete absence of hybridization signal with an apparently normal amount of DNA present in the lanes.

mentally retarded females (n=54) were cytogenetically positive. The mean fra(X) expression of these latter 2 groups was 14% (range 1%-32%) and 16% (range 2%-53%), respectively. DNA isolated from 55 females with the maternally inherited fra(X) gene was analysed for the CGG repeat fragment length. The hybridization pattern typical for this group of carriers is also shown in Figure 1.

In the EcoRI digest a single dose intensity is seen for the normal sized fragment, while the remainder of the signal is apparently dispersed over various fragments of 8.0 kbp and higher (Fig. 1a, lane 3). In the PstI digest a clearly dispersed signal is seen as well, but the normal fragment is less well defined (Fig. 1b, lane 3). In 14 cytogenetic positive female carriers with the maternal fra(X) gene, complete absence of the hybridization signal was found, indicating a very extensive dispersion of the fragment length. In 41 female

TABLE II: Mental Level in Cytogenetic Negative and Positive Females Carrying the Maternally Inherited Fra(X) Gene.

	Mental level			
	Normal[a]	Borderline[b]	Retarded[c]	Total
Cytogenetic Negative	10	0	0	10
Cytogenetic Positive	5	30	24	59
Total	15	30	24	69

a: No obvious signs of mental disabilities.
b: Mildly affected females, cognitive ability can occasionally be in the normal range, but significant learning disabilities were always present. They are self-supporting in society.
c: Moderately affected females, not self-supporting in society and in some cases institutionalized.

carriers a fragment of increased length, ranging in size from 1.2 kb to 6.5 kb, was found (Table III). To investigate whether there was a relation between the frequency of cytogenetic expression and CGG repeat length, a Pearson correlation test was performed for this group. A statistically significant positive correlation ($r = 0.43$, $P < 0.01$) was found between the level of fra(X) expression and CGG repeat length (Fig. 2).

DISCUSSION

We show here that the increase of the CGG repeat length in female carriers with the paternal fra(X) gene varies from 0.2 - 0.6 kb and is mostly seen as a homogeneous band on Southern blots. However, in most carrier females with the maternal fra(X) gene a dispersed hybridization signal with an apparent length increase in the range from 0.2 - 5.5 kb is observed. Our data are in general agreement with the classification proposed by Oberlé et al. (1991) in that 2 distinct classes of fra(X) mutations can be observed, namely the short inserts and the much larger and dispersed inserts. The present study confirms that the origin of the fra(X) gene is a major determinant for the cytogenetic expression and mental status of the female carriers. In particular,

TABLE III. Distribution of CGG Repeat Length in Cytogenetic Negative and Positive Females Carrying the Maternally Inherited Fra(X) Gene

	CGG Repeat Length in Kbp (PstI fragment)				
	Normal[a] fragment	Increased[b] fragment	Defined[c] smear	Absence[d] signal	Total
Cytogenetic Negative	0	4	2	0	6
Cytogenetic Positive	0	0	35	14	49
Total	0	4	37	14	55

a: Uniform fragment of normal length and normal intensity (1.0 kbp).
b: Uniform fragment of increased length and single-dose intensity, ranging in size from 0.2-0.4 kbp.
c: Multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards. In this sample the fragment were only slightly dispersed over a range of 1.4-6.5 kbp.
d: Complete absence of hybridization signal with an apparently normal amount of DNA present in the lanes.

all females who carry the paternal fra(X) gene are mentally normal and demonstrate no cytogenetic expression or only at a very low frequency, which is in agreement with Sherman et al. (1985).

In females who inherited the maternal fra(X) gene, cytogenetic expression appears to be linked with the mental status. While all mentally borderline and retarded women were fra(X) positive, only a third of the mentally normal females showed fra(X) expression. The level of fra(X) expression between mentally retarded and borderline female carriers is not significantly different (Wilcoxon, $P > 0.68$), which is in agreement with other reports (Sherman et al., 1985; Borghgraef et al., 1990; Grigsby et al., 1990; Bell et al., 1991). Yu et al. (1991) have reported that there is no obvious direct relationship between the CGG repeat length and mental status. Recently, Rousseau et al. (1991) reported that 53% of the female subjects in which the repeat sequence

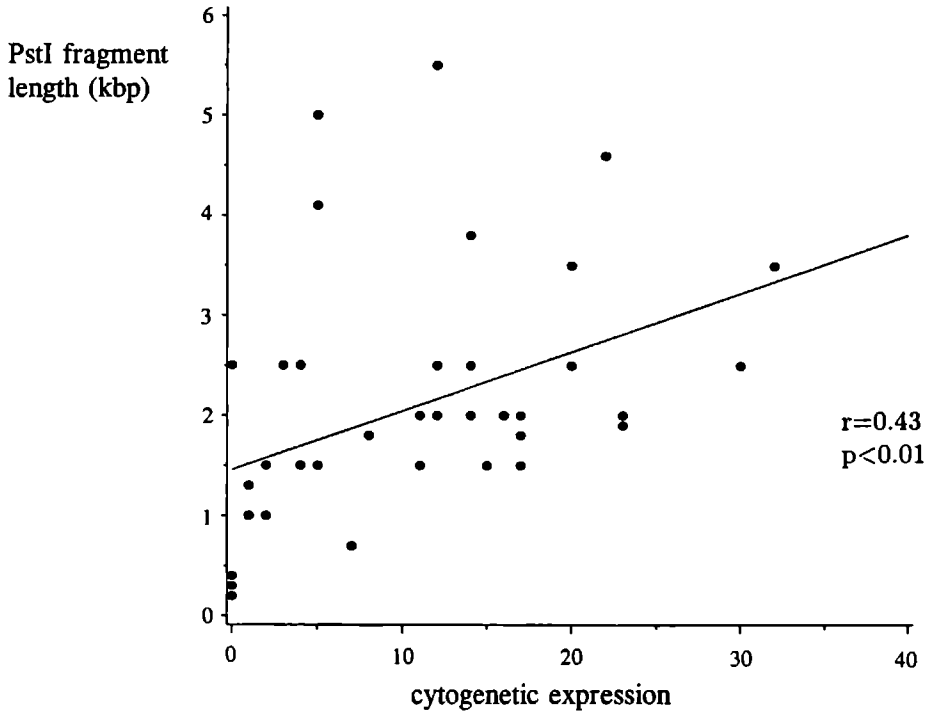

Fig. 2. Linear regression of the relation between increased PstI fragment length (uniform fragment of increased length and multiple recognizable bands from which the smear developed upwards) and cytogenetic expression in females, carrying the maternally derived fra(X) gene (n=41).

was at least a defined smear (full mutation) were mentally impaired. We have found in 14 cytogenetic positive female carriers with the maternal fra(X) gene a complete absence of hybridization signal, indicating extensive dispersion of the CGG repeat. None of these carriers was mentally normal. The possible correlation between mental status and repeat length in all carrier females needs to be evaluated in more detail.

The observed effect of the origin of the fra(X) gene on both the cytogenetic expression and the CGG repeat length suggests that both phenomena are causally related. Indeed, in the group of female carriers with the maternal fra(X) gene, we did find a statistically significant correlation (r=0.43)

between the level of cytogenetic expression and the CGG repeat length. Our observations confirm the preliminary results of Oberlé et al. (1991) and Yu et al. (1991), that the degree of increase of the CGG repeat length is associated with the frequency of cytogenetic expression and lends further support to the hypothesis put forward by Kremer et al. (1991) that the amplification of the CGG repeat causes the cytogenetic instability.

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CHAPTER 7

VALIDATION OF LINKAGE-BASED DNA-DIAGNOSIS OF FRAGILE X GENE CARRIERS WITH THE CGG REPEAT PROBE.

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SUMMARY

We have evaluated our carrier testing for the fragile X (fra(X)) syndrome, which was based on linked DNA markers, with the direct analysis of the CGG repeat sequence in the fra(X) gene. PstI and EcoRI blots were hybridized with a probe derived from the region just 3' of the CGG repeat in Xq27.3. We found the mutation analysis to be very sensitive as all 71 obligate gene carriers as well as 135 fra(X) patients tested showed evidence for an increased restriction fragment length encompassing the CGG repeat sequence with or without dispersion of the hybridization signal (mosaicism). Based on linked DNA markers, 6 out of 50 cytogenetic negative and mentally normal males at risk and 15 of 72 females at risk had inherited the allele at risk. All of these diagnoses could be confirmed by analysis of the CGG repeat length.

INTRODUCTION

Counselling of families in which the fragile X (fra(X)) syndrome segregates is problematic as female but also male carriers of the fra(X) gene may be without any symptoms. Cytogenetic analysis of the fragile site at Xq27.3 has its limitations as it has become clear that the expression of the fragile site in mentally normal gene carries is mostly negligible (Smits et al., 1992). In the past we have employed linked DNA markers for the identification of asymptomatic gene carriers and have offered this test as an integral part of our genetic service. Now that the fra(X) gene has been cloned (Verkerk et al., 1991), and is associated with an expansion of a CGG repeat sequence (Oberlé et al., 1991; Yu et al., 1991; Kremer et al., 1991; Fu et al., 1991), direct mutation analysis is possible based on the indirect analysis of the expansion of the CGG repeat in the 5'region of the FMR-1 gene. We have reanalyzed our family material for this CGG repeat sequence and were able to classify correctly patients, asymptomatic obligate gene carriers, as well as to confirm our carriership predictions based on linked DNA markers.

MATERIAL AND METHODS

Linkage Analysis

Fra(X) families were diagnosed cytogenetically and tested for restriction fragment length polymorphisms essentially as described before (van Oost et al.,1991). The polymorphic markers used for linkage were pRN1 (DXS369)

(Oostra et al., 1990), pJH89 (DXS539) (van den Hurk et al., 1991), pVK23B (DXS297) (Suthers et al., 1990), RS46 (DXS548) (Riggins et al., 1992), VK21A and VK21C (DXS296) (Suthers et al., 1989), II-10 (DXS466) (Hulsebos et al., 1991) and pU6.2 (DXS304) (Dahl et al., 1989). Risks for carriership for the consultands were calculated with the MLINK program of the LINKAGE package version 5.03 (Lathrop et al., 1984). Penetrance of the cytogenetic aberration in males and females were estimated to be 0.9 and 0.64, respectively and the gene frequency was taken as 0.001.

Direct Mutation Analysis

The probe used for the detection of the fra(X) mutation was a 600 bp XhoI-PstI subclone derived from the 5.1 kbp EcoRI fragment (pAO365) which encompassed the CGG-repeat sequence (Verkerk et al., 1991). Under standard hybridization and washing conditions (van Oost et al., 1991) a clear single copy signal could be obtained with this probe in control individuals.

The PstI and EcoRI fragment sizes were classified as follows: (i) uniform fragment of normal length and normal intensity (either the 1.0 kbp PstI or the 5.1 kbp EcoRI fragment), (ii) uniform fragment of increased length and a single-dose intensity, (iii) multiple recognizable bands of reduced intensity identified by the fragment size from which the smear developed upwards, (iii) complete absence of hybridization signal with apparently normal amount of DNA present in the lanes. The amount of DNA on the blots was estimated from the ethidium bromide stain and was of particular importance in the estimation of a double- or single dose intensity of the normal PstI or EcoRI fragment in women.

RESULTS

Six hundred individuals from 38 families in which the fra(X) gene was segregating were tested with the CGG repeat probe. These families were tested before with a number of markers on both sides of the fra(X) gene, in order to assess the carrier status of relatives at risk. In Fig. 1 a fra(X) family (285/FX) is shown which was analyzed for the length of the CGG-repeat encompassing PstI and EcoRI fragments. In the grandmother, who was an obligate carrier based on genealogic data, a normal sized band and a band of increased size was seen. In the PstI digest the increased fragment was predominant, while for the EcoRI digest both bands seemed to be of equal

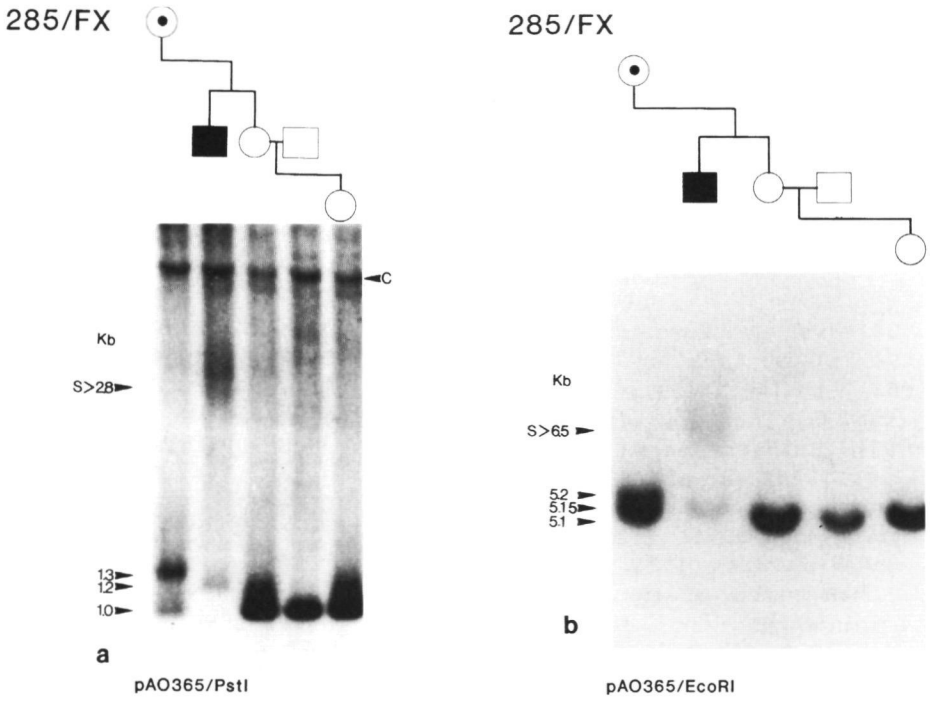

Fig. 1. CGG-repeat analysis in a fra(X) family. PstI (a) and EcoRI (b) blots were incubated with the pAO365 probe, which hybridizes with the PstI and EcoRI fragments which encompasses the CGG repeat sequence in the FMR-1 gene. In the margins the approximate lengths of the restriction fragments are given. C: constant fragment; solid symbols: mental retardation and cytogenetic expression; obligate carriers have center circles.

intensity. In her affected son most of the hybridization signal is dispersed over fragments at least 1500 bp increased in size. However, it is clear that part of the hybridizing fragments for this patient is only moderately increased in size and, in effect, is smaller than the length of the increased fragment seen in the mother. Both her daughter and her grand-daughter showed perfectly normal hybridization signals for EcoRI as well as PstI. As the grandfather was deceased, DNA-linkage analysis based on the flanking

markers DXS369 and DXS296 was only informative for the granddaughter. The haplotype of the X-chromosome which she inherited from her mother was, for these markers, completely different from that of her affected uncle. In Fig. 2 a typical fra(X) family is shown with the sibships of 3 daughters of a normal transmitting male (identified by extendend pedigree analysis). Both patients displayed a dispersed PstI and EcoRI fragment, while in their

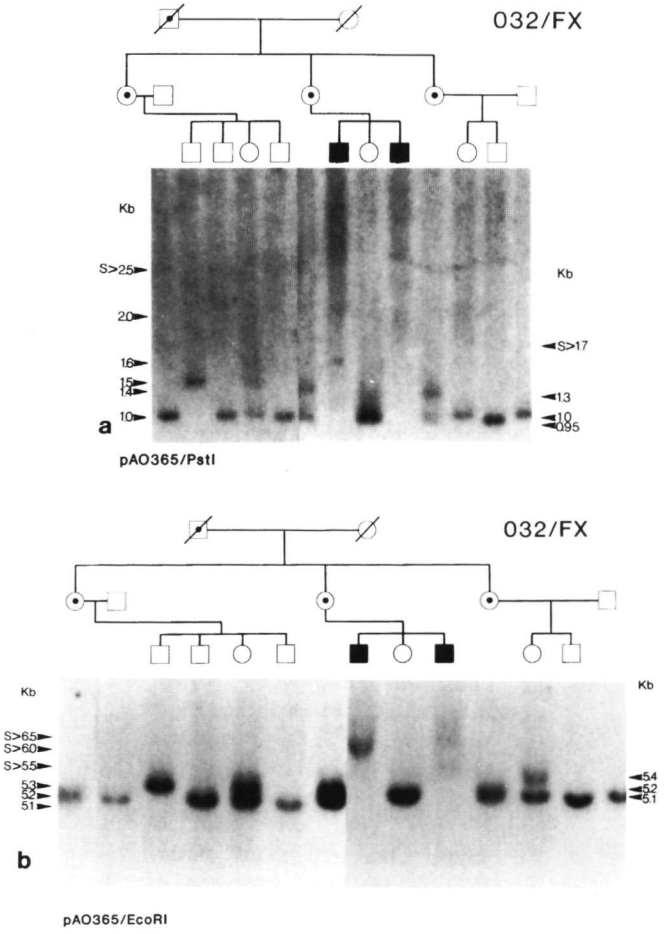

Fig. 2. CGG-repeat analysis in a fra(X) family with a normal transmitting male. For legend see Fig. 1.

mothers the size of the PstI and/or EcoRI band was increased 100-400 bp. However, also in 2 phenotypically normal granddaughters and one normal grandson the PstI and/or EcoRI fragments were 100-400 bp increased, or even dispersed as is seen for the daughter of the youngest carrier mother. This family was previously typed for the CA repeat RS46 (DXS548). For all grandchildren the predicted carrier status was in agreement with the direct analysis of the CGG repeat length. In the following the analysis of all family data are summarized (Table I).

TABLE I. Distribution of CGG Repeat Length for Fra(X) Patients, Carriers and Control Males and Females.

	CGG Repeat Length in Kb (PstI fragment)				
	Normal[a] fragment	Increased[b] fragment	Defined[c] smear	Absence[d] signal	Total
Male patients	0	0	57	29	86
Female patients	0	0	34	15	49
Male carriers	0	11	0	0	11
Female carriers	0	52	5	1	58
Control males	53	0	0	0	53
Control females	22	0	0	0	22
Total	75	63	96	45	279

a: Uniform fragment of normal length and normal intensity (1.0 kbp).
b: Uniform fragment of increased length and single-dose intensity, ranging in size from 1.2-1.6 kbp.
c: Multiple recognizable bands of reduced intensity identified by the fragment size from which the
smear developed upwards. The fragment size from which the smear developed was 1.4-9.0 kbp.
d: Complete absence of hybridization signal with an apparently normal amount of DNA present in
the lane in males. In females a normal sized band of single dose intensity was seen only.

Repeat Length in Control Persons

Repeat length in normal individuals was assessed in the family members related by marriage. For all 53 males and 22 females studied no increased PstI and/or EcoRI fragments were seen. In the PstI digests slight variations in fragment sizes were noted in the order of 50 basepairs or less. In females the PstI signal was often not discrete but presented itself with a band width of about 50 bp with a apparently monotonous decrease in intensity. In no instance aberrant fragment sizes were noted in the family members not at risk.

Repeat Length in Mentally Retarded Patients

In all 135 mentally retarded, cytogenetically positive, fra(X) patients studied no normal sized PstI and/or EcoRI fragments were seen. All patients showed in PstI digests evidence for a very dispersed signal and in 33% no hybridization signal at all was seen. This was taken as evidence that the repeat was dispersed beyond the detection limit and was confirmed on the basis of the fact that a normal amount of DNA was present on the blot while other lanes on the same blot gave clear positive hybridization with the repeat probe. In all but 2 male patients an EcoRI fragment of defined increased size was seen, probably a reflection that the dispersed fragments were more compressed after electrophoresis of EcoRI digests compared to PstI digests. Likewise, in 4 female patients, who had received the grandpaternal allele, an EcoRI fragment of defined increased size was seen.

Repeat Length in Mentally Normal Obligate gene Carriers

Eleven male carriers were identified through extended family studies. In all a fragment of defined increased size was observed for PstI and/or EcoRI. For the 58 identified female carriers the repeat length depended clearly on the origin of the fra(X) chromosome. Most female carriers showed a PstI fragment of defined increased size, while in some a dispersed signal was seen (Smits et al., 1992).

Repeat Length in Relatives With a Low Risk Based on Linkage Analysis

Forty-four males and 57 females at 25 or 50% a-priori risk were found to

have a residual chance of being a carrier of less than 10% based on the analysis of linked DNA-markers (Table II). In all of these consultants this negative result was confirmed by the absence of any aberration in either the PstI and/or EcoRI digest.

TABLE II. Validation of Linkage-Based DNA-Diagnosis of Fra(X) Gene Carriership.

	Risk based on linkage analysis[1]	EcoRI and/or PstI fragment[2] Normal	Increased
Males	1%	40	0
	4%	4	0
	99%	0	6
Females	1%	46	0
	3%	1	0
	4%	5	0
	5%	5	0
	90%	0	1
	99%	0	14

1. Risks were based on phase-known meioses informative for linked DNA-markers and calculated with the MLINK program of the LINKAGE package.
2. EcoRI and PstI blots were hybridized with the probe just 3' of the CGG repeat and the autoradiograms were scored as described in the Material and Methods section

Repeat Length in Relatives With a High Risk Based on Linkage Analysis

Six males and 15 females at 25-50% risk were found to have inherited the allele at risk based on linkage analysis. In all of these cases this positive result was confirmed by the presence of either an increased PstI and/or EcoRI fragment of defined size. In none of these non-manifesting carriers evidence for dispersed fragments was obtained.

DISCUSSION

Counselling of fra(X) families has posed a vexing problem, as many of the female as well as the male fra(X) gene carriers are phenotypically normal. Linkage-based DNA-diagnosis has been applied initially with markers localized relatively far from the disease gene locus (Brown et al., 1988). In particular, the limited informativity of these restriction fragment length polymorphisms has made the necessary application of flanking markers cumbersome (Mulley et al., 1987). The recent development of tightly linked CA-repeat polymorphisms have ameliorated these diagnostic problems significantly (Riggins et al., 1992; Richards et al., 1992).

However, direct analysis of the gene defect is always preferable in particular in families in which the diagnosis is uncertain and/or in which no patients are available for study. This has been demonstrated recently by mutation analysis in the fra(X) syndrome (Rousseau et al., 1991; Sutherland et al., 1991; Nakahori et al., 1991). In this paper we were able to confirm that the expansion of the CGG repeat, as measured by the length of the PstI and/or EcoRI fragment, is sensitive and specific. By using the 2 enzymes EcoRI and PstI, we were able to score aberrations in all patients, in contrast to all of the control individuals. PstI is particular by well suited for the identification of normal transmitting males and their carrier daughters. EcoRI, in turn, is more suited for analysis of the dispersion of the repeat as is seen in male and in female patients. Also, analysis with both restriction enzymes will be of help in the evaluation of possible artifacts as is exemplified by the pedigree in Fig. 1. However, our classification of the variation in the CGG repeat length remains tentative. Transitions between the different forms might be present as is seen in the patient in Fig. 1. Detailed and more sensitive analysis is needed to evaluate all the different size distributions for their diagnostic impact, as was also pointed out by Rousseau et al. (1991).

As positive identification of patients seems straightforward, exclusion of the syndrome is less so. In normal individuals slight variations of the PstI fragment length were noted frequently. Together with the often observed slight smearing of the PstI fragment in normal individuals made this the definite exclusion of carrier status problematic. Direct measurement of the CGG repeat on both X-chromosomes and identification of heterozygosity might be of help in the exclusion diagnosis. Linkage analysis, in particular with the highly informative CA-repeats, is likely to keep its own merit in exclusion-diagnosis. We also want to point out here that we find the aberrations in the repeat length in patients with the full Martin-Bell

phenotype. In a fra(X) positive family in which the mental retardation and facial characteristics were less pronounced, we did not find any evidence for expansion of the CGG repeat (unpublished data). Thus, caution should be exercised in direct analysis of the CGG repeat in families in which not all the typical facial, mental, and cytogenetic abnormalities occur together.

By the retrospective analysis presented in this paper we were able to show that our initial linkage-based DNA diagnoses were correct for all consultands. The risk figures could all be updated to almost certainty. As the CGG-repeat expansion could also be found in fetal material, the CGG repeat analysis will be the method of choice for postnatal as well as prenatal diagnosis of the fra(X) syndrome.

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CHAPTER 8

PREDICTION OF MENTAL STATUS IN CARRIERS OF THE FRAGILE X MUTATION USING CGG REPEAT LENGTH

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SUMMARY

For genetic counseling in fragile X (fra(X)) families it is important to know the diagnostic impact of the CGG repeat length in carriers of the fragile X mutation. We have analyzed the CGG repeat length in 106 males and 73 females who had inherited the maternal fra(X) mutation. The sensitivity, specificity, and the presence of the pre/full mutation for predicting mental retardation as measured on Southern blots of PstI/EcoRI digests, were calculated. In males the sensitivity, specificity and negative predictive value were 99%, 100% and 94%; respectively. In females the specificity (60%) and, consequently, the positive predictive value (82%) was reduced. Therefore, it remains impossible to predict accurately whether a female fetus demonstrating a full mutation will be affected. On the other hand, the negative predictive value of the CGG test in females was 100%. We have no evidence, as yet, that for female carriers with the full mutation cytogenetic analysis is of additional value to predict the mental status. However, we have observed one mentally retarded fra(X) case with extreme mosaicism in which a typical premutation fragment was the predominant DNA species. Therefore, until more data and better DNA tests are available, we would like to advocate additional cytogenetic investigation if in a fetus a CGG repeat length in the premutation range is found.

INTRODUCTION

Fragile X (fra(X) syndrome, a relatively common type of X-linked mental deficiency, is caused by an expansion of a trinucleotide repeat p(CGG)n at the *FMR1* locus in the 5' region of the gene (Kremer et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Yu et al., 1991). This expansion may be expressed cytogenetically as a fragile site at the distal long arm of the mutated X-chromosome. It has been proposed that the length of the CGG repeat correlates with cytogenetic expression of this fragile site as well as with phenotype (Oberlé et al., 1991; Sutherland et al., 1991; Yu et al., 1991, 1992; Smits et al., 1992a). This implies that the variation in CGG repeat length has immediate diagnostic applications. In order to examine its diagnostic value, we have analyzed the CGG repeat length by Southern blot analysis of digested genomic DNA in a large cohort of carriers of maternally inherited fragile X mutation. The specificity, sensitivity, and predictive values of the DNA analysis were determined.

MATERIALS AND METHODS

Subjects

From the total offspring (n=347) of obligate fra(X) female carriers, we selected 73 females and 106 males who had inherited the FRAXA mutation. Inheritance was established through cytogenetic analysis, DNA linkage analysis or direct detection of the CGG repeat length and/or by pedigree analysis. Maternal transmission of the fra(X) mutation was initially traced by pedigree analysis and subsequently confirmed by DNA linkage analysis. Mental status of the selected individuals was obtained anamnestically and differentiated as normal, borderline, or retarded. Normal mental status implied that no obvious signs of mental disability were present. Mentally borderline individuals were mildly impaired as they had significant learning disabilities, but were self-supporting in society. Mentally retarded individuals were moderately affected and not self-supporting in society.

Estimation of the CGG Repeat Length by Southern Blot Analysis

For routine DNA-diagnosis of the FRAXA mutation we assessed the length variation of the CGG repeat by Southern blot analyses of PstI/EcoRI restricted total genomic DNA. In atypical cases in which the genotype to phenotype correlation was not consistent, we employed a BglII digest too, to determine the extremely dispersed CGG repeats. The probe used for the detection of the fra(X) mutation was a 600 bp XhoI-PstI subclone derived from the 5.1 kbp EcoRI fragment (pAO365) which encompasses the CGG repeat sequence (Verkerk et al., 1991).

The PstI and EcoRI fragment sizes were recorded and classified as follows: (i) uniform fragment of normal length and normal intensity, (ii) uniform fragment of increased length in the premutation range, (iii) heterogeneous fragments above the premutation range or complete absence of hybridization signal with a normal amount of DNA present in the lane, indicating the presence of a full mutation. Only Southern blots with at least 15 different individuals were tested. This allowed us a reliable estimate of the dosis on the autoradiogram. The amount of DNA actually present on the blot was estimated on a comparative basis from the Ethidium Bromide staining in the gel as well as from the back-ground hybridization throughout the lanes on the autoradiogram. In our hands this method was equal or superior to comparison with a so-called "control probe". This conclusion was reached after numerous multiple dosis determinations (data

not shown).

RESULTS AND DISCUSSION

Relation Between CGG Repeat Length and Phenotype

The results of the length increase of the PstI/EcoRI fragments in fra(X) mutation carriers relative to sex and mental status are summarized in Table 1. In case the hybridization signal for PstI was absent (22 males and 15 females), EcoRI digests showed in all but one (see below) a heterogeneous smear in the full mutation range.

TABLE I. Length Increase of the Restriction Fragments Encompassing the CGG Trinucleotide Repeat in Fra(X) Gene Carriers Relative to Sex and Mental Status

		Length increase (kbp) of PstI/EcoRI restriction fragment encompassing the CGG trinucleotide repeat	
Group	Mental status	Premutation	Full mutation
Males	Retarded	1	90
	Normal	15	0
Females	Retarded	0	21
	Borderline	0	27
	Normal	15	10

Normal fragment length. No normal sized fragment was observed in any of the tested male carriers. Similarly, all tested female carriers demonstrated an increased fragment size, representing the abnormal X-chromosome, besides a size of normal fragment.

Premutation. All but one individual with a fragment length in the premutation range were mentally normal (n=31). The 26 cytogenetically tested individuals from this group (13 males and 13 females) did not express the fragile site. In the single mentally retarded male who showed only PstI/EcoRI fragment sizes in the premutation range, 7% of his cultured lymphocytes were positive for the fragile X chromosome. In an effort to solve this discrepancy we analyzed also a BGlII

digest of this patient. In typical fra(X) patients a distinct restriction fragment of increased sizes is seen (fig. 1, lane 5). In this patient a smear was seen for the BglII digest (fig. 1, lane 2,3,4) which indicated an extremely dispersed expansion in about 20% of his X-chromosomes.

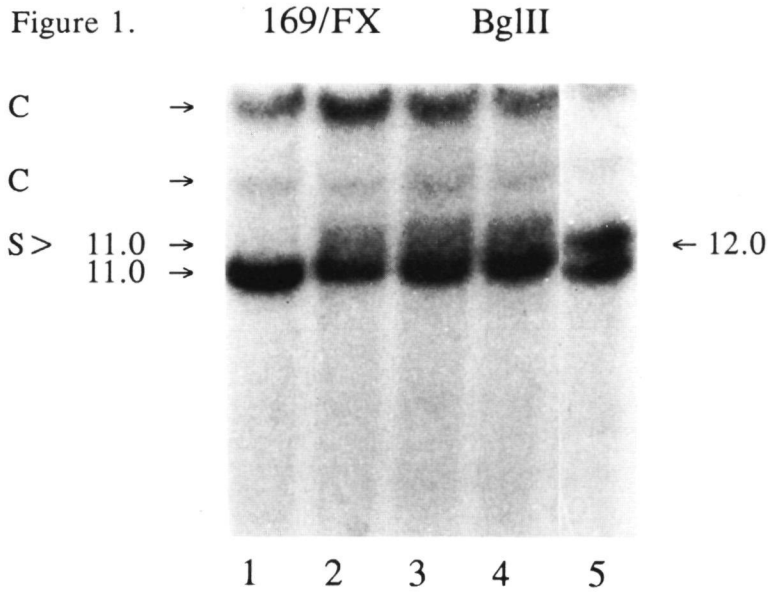

Fig. 1. BglII-Southern blot analysis in a male FRAXA patient with extreme mosaicism. The autoradiagram of the BglII-Southern blot shows in **lane 1** the DNA fragment length of a normal male and in **lane 5** of a mentally impaired female carrier with 3% cytogenetic expression of the fragile site. Both controls are compared to the patient DNA with 3 different DNA concentrations (**lane 2** 7.5 μg, **lane 3** 11.25 μg, **lane 4** 15 μg). The patient DNA shows a faint heterogeneous smear in contrast to the carrier female (lane 5), who shows a discrete band. C= bands are due to back-ground hybridization and are seen in all controls and patients. S= smear of fragments.

Full mutation. Heterogeneous fragments in the full mutation range were present in all but one (see previous paragraph) mentally retarded male (n=91). Although this was an exceptional case, as mentioned before, prudence is called for when determining the repeat length based only on PstI/EcoRI digest. All mentally

impaired females (n=48) showed the full mutation. Of the 46 mentally retarded women who were tested cytogenetically, all but one showed fragile X expression. In contrast with males, 10 of the 58 females who carried a full mutation were mentally normal. Seven of them were cytogenetically tested and 5 showed fragile X expression.

Predictive Value of the CGG Repeat Length Determination

We have calculated the sensitivity, specificity, as well as the positive and negative predictive value encompassing the CGG trinucleotide repeat length, using the collected DNA data of PstI/EcoRI digests (table 2). Sensitivity was calculated from the proportion of mentally impaired carriers who were classified correctly by the presence of a full mutation. In males as well as in females, this sensitivity of the CGG trinucleotide repeat length determination was very high (Table 2).

TABLE II. Sensitivity, Specificiy, Positive Predictive Values (pVpos), and Negative Predictive Values (pNeg) of CGG Repeat Length Determination for Fra(X) Syndrome, Using PstI/EcoRI Restriction Enzymes

	Percent			
Sex	Sensitivity	Specificity	pVpos	pVneg
Male	99	100	100	94
Female	100	60	82	100

The specificity of the CGG-repeat test for mental status is determined by the proportion of mentally normal carriers who are correctly classified by the presence of a premutation. Our calculations show that the specificity of the CGG repeat test for male carriers is maximal (100 percent) in contrast with female carriers (60 percent). Our data are in agreement with the results published by Rousseau et al. (1991) that 100 percent of males who carry the full mutation will be mentally impaired. In accordance with Rousseau et al. (1991), Mulley et al. (1992) and Yu et al. (1992), we established that females with a full mutation can be either normal carriers or mentally affected.

If these figures for specificity and sensitivity are to be presented as positive and negative predictive values the frequency of mental retardation in the target population (fra(X) carriers) must be specified. We used the previously derived penetrance value of 85% and 64% for males and females, respectively (Smits et al., 1992b) and applied the rule of Bayes. The positive predictive value in males was 100% and in females only 82%. As 2 categories of mentally impaired females were assessed, borderline and retarded, we calculated the positive predictive values for both categories. Given the 26% penetrance of mentally borderline and 38% penetrance in retarded females (unpublished data), positive predictive values of 33% (26/64 x 0.82) and 49% (38/64 x 0.82) were obtained, respectively. Therefore, it remains impossible to predict accurately whether and to which degree a female fetus with a full mutation will be affected. In contrast, the negative predictive value of the CGG repeat determination in females is 100%. This implies that a prenatally diagnosed fetus of the female sex, with a small increase in fragment size (premutation), will have less than 1% percent chance to be mentally affected. The presence of one mentally retarded male out of a group of 91, who initially showed a premutation using a PstI/EcoRI digest, illustrated that in a case of extreme mosaicism it is difficult to visualize a smear typically for an affected individual. Since this retarded male showed fragile X expression, it makes the point that cytogenetic testing in cases of a CGG repeat length in the premutation range may give additional information about the mental status of the carrier. Our data confirm the results of others that, for optimal detection of a possible full mutation, the use of BglII digest is useful (Rousseau et al., 1992; Knight et al., 1992; Oostra et al., 1993). However, to date the number of directly diagnosed patients showing an extreme mosaicism on BglII digest is not known. Therefore, until more data are available, we would like to advocate additional cytogenetic investigation if a fetus carries a premutation. We have no evidence as yet, that in fetuses with a CGG repeat length in the full mutation range, cytogenetic analysis is of additional value to predict the mental status.

Thus, direct molecular analysis of the CGG repeat length together with PCR-based genotyping of flanking CA repeats is now the strategy of choice for prenatal fra(X) diagnosis. Special care has to be given if a premutation is detected. In those cases DNA analysis should be supplemented with cytogenetic analysis to exclude extreme mosaicism of the fra(X) mutation, which could lead to a false negative prediction of the phenotype.

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CHAPTER 9

NORMAL PHENOTYPE IN TWO BROTHERS WITH A FULL *FMR1* MUTATION

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SUMMARY

The fragile X (fra(X)) syndrome is associated with an expanding CGG repeat in the 5' untranslated region of the first exon of the *FMR1* gene. Subsequent methylation of the promoter region inhibits expression of the *FMR1* gene. In two clinically normal brothers large, expanded CGG repeats and cytogenetically visible fragile sites were found. The *FMR1* promoter was unmethylated and both RNA and protein could be detected. This indicates that inactivation of the *FMR1* gene and not repeat expansion itself results in the fra(X) phenotype. We conclude that repeat expansion does not necessarily induce methylation and that methylation is no absolute requirement for the induction of fragile sites.

INTRODUCTION

Triplet repeat expansions have been associated with a variety of genetic disorders (1-7) and fragile sites (8-11) or both (8, 9). Limited expansion of CAG repeats within the coding region of genes cause a number of neurodegenerative disorders, probably due to a gain-of-function of the protein. Very large, expanding repeats are found in myotonic dystrophy (2) and in a number of fragile sites (8-11). As yet, the destructive mechanism of the expanded CTG repeat in the 3' untranslated region (UTR) of the DM-kinase gene is unknown and conflicting reports on over- or underexpression of the gene product in patients exist (12, 13). The triplets involved in fragile sites are CGG or CCG and clinical features can be present (FRAXA, FRAXE) or absent (FRA16A, FRAXF). The best characterized example is the FRAXA-site at Xq27.3 (14, 15), indicative of the fra(X) syndrome, which is the most common form of hereditary mental retardation in males (16-18). The disease is associated with expansion of a CGG repeat, located in the 5'UTR of the *FMR1* gene. Methylation of the *FMR1* promoter region silences the gene (19-23) and absence of the *FMR1* protein results in the clinical phenotype. This is supported by a rare group of patients showing the clinical features of the fra(X) syndrome, but who were cytogenetically negative. In these patients deletions (24-26) or a point mutation (27) in the *FMR1* gene were demonstrated, which resulted in the absence of or a defective *FMR1* protein.

So far, several groups have tried without success to find a correlation between the number of CGG triplets and the severity of mental retardation (28, 29). It turned out that the methylation status of the *FMR1* promoter was the most

correlated parameter in male patients (30). For example, mild phenotypic symptoms have been reported for patients with an only partially methylated full mutation, but without cytogenetic FRAXA expression (31, 32). Furthermore, incomplete and complete absence of methylation was found in a group of high functioning male patients with cytogenetic expression. In this group the presence of *FMR1* protein was demonstrated, although in reduced amounts (33). It is, however, unclear if this is caused by a lower gene expression or by a translation suppression due to the length of the CGG repeat.

The expanded CGG or CCG repeats are predicted to undergo conformational changes, which induce *de novo* methylation (34), thereby stabilizing the unusual structures, probably to mark them for repair (35). These structures could suppress transcription and replication of the *FMR1* gene and so induce cytogenetic expression (35). The familial case described in this paper of two completely normal brothers with CGG repeat expansion and cytogenetic FRAXA expression may provide a clue to both the mechanism of inhibition of *FMR1* expression and induction of fragile sites.

RESULTS

Analysis of the Fra(X) Locus

The fra(X) family was ascertained through a mentally retarded boy, born in 1980 (Fig. 1, 7316). Cytogenetically, 10% FRAXA-site expression was found. Further DNA analysis with PstI and EcoRI digested DNA from peripheral blood lymphocytes (PBL) revealed expansion of the CGG repeat with fragments ranging from apparently normal to full mutation (up to 1500 repeats; Fig. 1a+b). A mosaic pattern from premutation to full mutation is found in 20% of the fra(X) patients (30, 32, 36), but the pattern of this patient is very rare. The mother of the proband had a premutation and a normal allele (Fig. 1a, 7315). Subsequent family analysis revealed 5 more carriers, among whom were 2 males having both cytogenetic FRAXA expression and a full mutation by DNA analysis (Fig. 1a+b, 7298 and 7241). Surprisingly, these two males were phenotypically normal. In repeated experiments, we found 6 and 13% fragile site expression and large repeat expansions of 170-340 and 100-1500 copies, respectively (Fig. 1a).

In all mentally retarded patients with similar repeat sizes tested, the CpG island in front of the repeat was methylated as a result of which the gene was

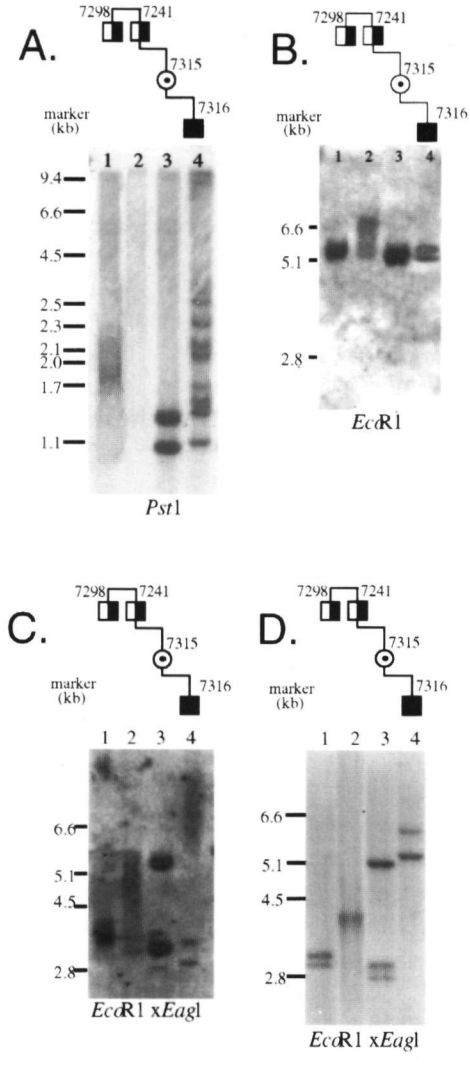

Fig. 1. Southern blot analysis of genomic DNA. PstI (A) and EcoRI (B) digestions of PBL DNA and EcoRI-EagI digestions of PBL (C) and EBV transformed blood lymphocyte DNA (D). Half-filled squares indicate males with normal phenotype, but with cytogenetic FRAXA expression. A filled square indicates a male fragile X patient with mental retardation and cytogenetic FRAXA expression. A dotted circle represents an obligate female carrier with a normal phenotype.

not transcribed. The methylation status of the *FMR1* gene was determined in this family using the enzymes EcoRI + EagI on DNA from PBL and EBV transformed blood lymphocytes (Fig. 1c+d). The mentally retarded boy showed complete methylation of the full mutation, while the premutation and normal bands were unmethylated in PBL DNA (Fig. 1c). In contrast, no unmethylated bands were detectable in DNA from EBV transformed cells (Fig. 1d). The expanded *FMR1* genes of the mentally normal men were entirely unmethylated, both in PBL (Fig. 1c) and in EBV transformed blood lymphocytes (Fig. 1d). As expected, the female carriers in the family showed partial methylation of both X chromosomes (Fig. 1c+d).

A difference in repeat length distribution was found in blood DNA (Fig. 1c) compared with the distribution found in DNA isolated from EBV transformed blood lymphocytes (Fig. 1d). DNA from the cell line of patient 7316 contained only two bands left from the full mutation range. Male 7298 had a repeat distribution with a tendency to the premutation range, while on the other hand the cell line of male 7241 showed an almost single repeat length of 400 repeats, which is completely in the full mutation range. This shows again that EBV transformed cell lines are not always a good representation of the variety of different cells with different repeat lengths found in blood and that they are the product of a limited number of cells. A difference in methylation between PBL and EBV transformed blood lymphocytes was only found for the patient 7316.

Expression of the *FMR1* Gene

Methylation of the *FMR1* promoter has been reported to inhibit expression of the gene. The expression of the *FMR1* gene in the three males with large repeat expansions was tested in peripheral and EBV transformed blood lymphocytes. As an internal control the level of *HPRT* transcription was determined, and classical fra(X) patients and controls were included for comparison. No *FMR1* mRNA was detectable in classical fra(X) patients with large repeat expansions and a methylated promoter region (data not shown). The expression in male 7316 was strongly reduced compatible with classical fra(X) patients. The level of *FMR1* mRNA in PBL was only slightly higher than in EBV transformed blood lymphocytes. The *FMR1* mRNA levels in males 7298 and 7241 were normal in both cell types.

We analyzed whether the large repeat had an influence on the (efficiency of) translation of the *FMR1* RNA. Immunoprecipitation was performed on equal

amounts of protein extracted from EBV transformed blood lymphocytes and FMRP (*FMR1* protein) was determined by Western blotting using anti-*FMR1* antibodies (22). The same passage of the culture was used as for the DNA studies. The *FMR1* protein was present in the cells from males 7298 and 7241 at a reduced level compared to normal controls (Fig. 2).

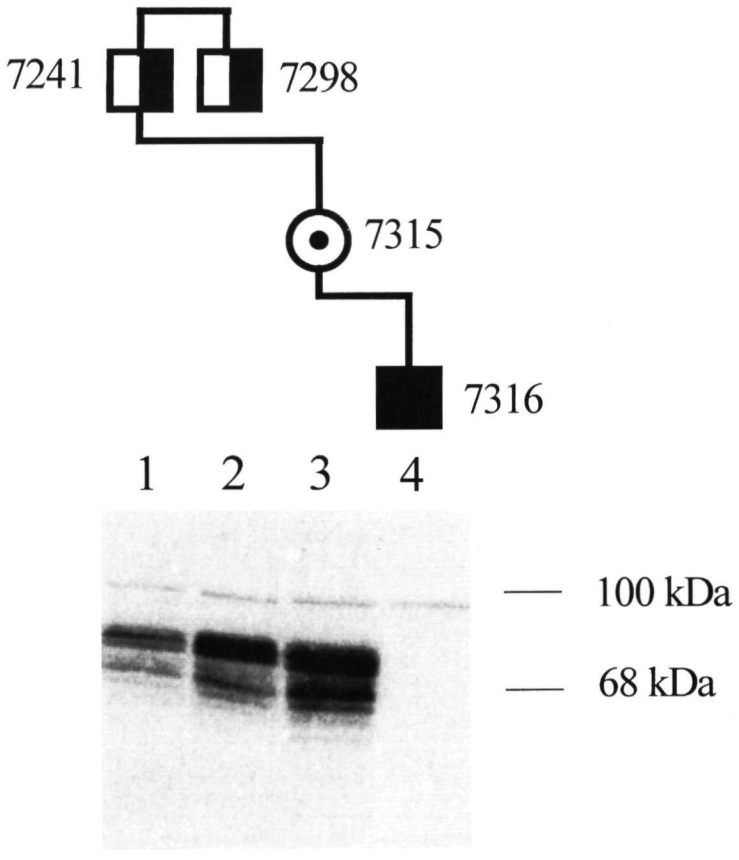

Fig. 2. Western blot analysis of FMRP in EBV transformed lymphocytes using polyclonal antibody α734. Pedigree symbols are as in figure 1.

The fra(X) male 7316 showed no *FMR1* protein at all, similar to classical fra(X) patients. His mother 7315 had a normal FMRP expression as might be expected from a carrier of a premutation.

To test whether the lower expression in these males was the result of expression in cells with a relatively small expansion and no expression in cells with a relatively large expansion, we tested the protein expression by immunocytochemistry on individual cells of the EBV transformed blood lymphocytes. Expression was found in all cells, but also at a lower level than in control cells (Fig. 3).

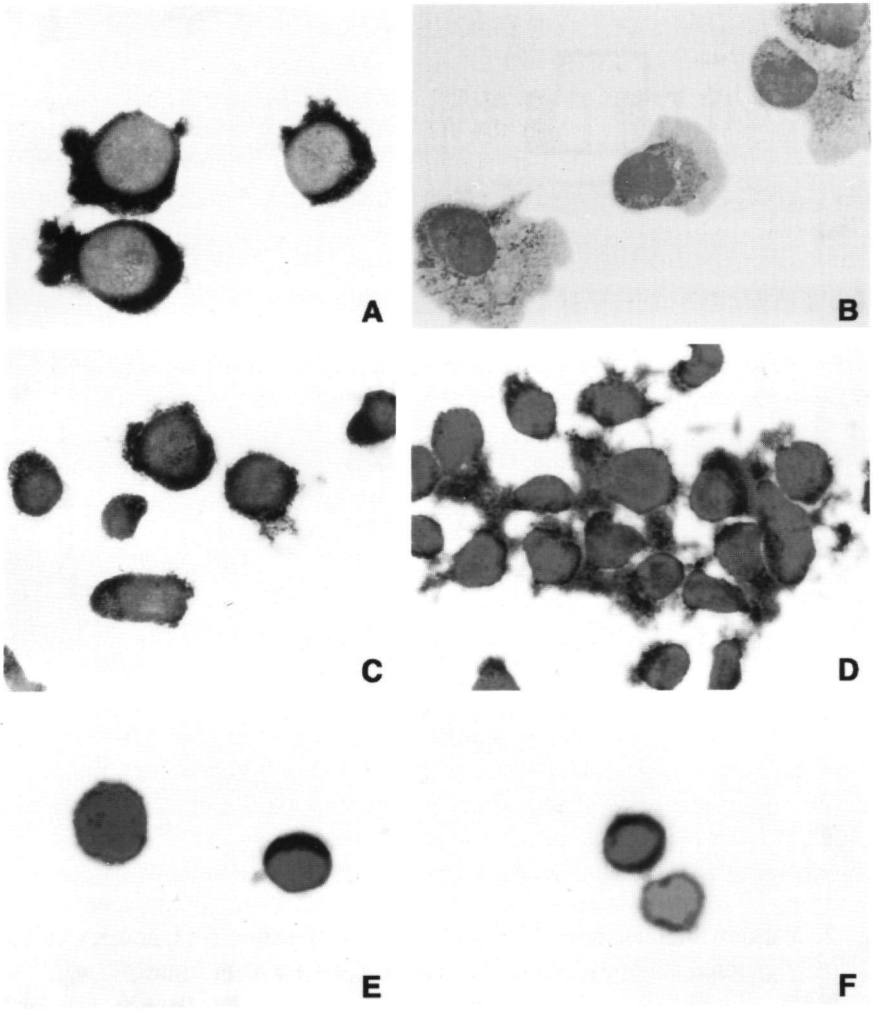

Fig. 3. Immunohistochemical detection of FMRP in EBV transformed lymphocyte and blood smears. Cell lines of control cells C49 (A), patient 7316 (B), individual 7298 (C) and individual 7241 (D). Blood smears of a control (E) and individual 7241 (F).

This means that a gene with a repeat length of 400 copies can be transcribed and translated (Fig. 1b). To evaluate the result of the cell lines, the expression of the *FMR1* protein in blood was analyzed directly on a blood smear of male 7241. Again all cells did show expression of the *FMR1* protein, but again at a level lower than control cells (Fig. 3).

DISCUSSION

In this study evidence is presented that expansion of the CGG repeat in the *FMR1* gene itself, even in the full mutation range, does not cause the fra(X) phenotype. It is the first example of a large, unmethylated expanded CGG repeat without any clinical consequences. This familial case might be exceptional, although it is unknown how many people in the general population may carry large repeat expansions. The family was analyzed, because of the presence of a mentally retarded grandson, but would have been missed otherwise. Even this grandson lacked the physical features of fra(X). But despite an extraordinary mosaic CGG repeat pattern, *FMR1* mRNA and protein levels in PBL were comparable with classical fra(X) patients.

Variation in severity of mental retardation has been shown previously for individual patients with different methylation patterns. Rousseau et al. (31) described a male with a full mutation with 40% methylation of the *FMR1* gene; unfortunately, protein expression was not determined. Hagerman et al. (33) described three high functioning fra(X) males that had unmethylated large repeats and showed reduced protein expression compared with control values. They did not show whether normal expression was found in a limited number of cells or a reduced expression was found in all cells. Our data support the conclusion that a minimum amount of protein is necessary for a normal phenotype; it could be speculated that this minimal amount may be necessary in every cell or at least in a high percentage of cells. This could explain that some of the females with a full mutation are normal and some are mentally retarded, although less severely than males with a full mutation, due to a skewed inactivation of the normal X chromosome. The amount of protein made as shown by immunohistochemistry was lower in blood and in EBV transformed blood lymphocytes in the males with the unmethylated full mutation compared with controls. However, Feng et al. (37) have shown that the amount of *FMR1* protein using ß-Tubulin as an internal control can differ by a factor of 2. Although the relation between RNA/protein levels and clinical status has not been extensively analyzed, it is evident that these parameters are most indicative for the expression of the *FMR1* gene and, most

likely, are best suited for clinical predictions.

Our data show that there is no obligatory relation between repeat expansion and methylation of the *FMR1* gene. This has already been demonstrated for normal transmitting males with relatively short repeat expansions (50-200 CGG repeats), but usually larger expansions lead to complete methylation of the *FMR1* promoter region. Only a few examples have been reported with incomplete or mosaic methylation patterns, providing supporting evidence that expansion and methylation can be uncoupled (31, 33). Sutcliffe et al. (20) have shown in chorionic villi of a male fetus with a full mutation that no methylation of the CpG island was found and that the *FMR1* gene was transcribed. This again indicates that in absence of methylation, transcription of a gene with a full mutation is possible. In the two brothers described in this paper, this uncoupling is absolute. Because the mechanism and enzymes involved in *de novo* methylation in humans are still unknown, one can only speculate about the factors involved. First, the length of the expanded repeat may be a factor. Short expansions do not cause methylation, but large expansions in general do. The family described here and the data from others (20, 38) showed that no absolute point exists at which methylation takes place, but that there may be a gradual scale with intra- and interfamilial variation. Secondly, our data show that additional (genetic) factors must play a part in the methylation process. In the two brothers no methylation is apparent, which means that either the specific methylase is defective or that the recognition site(s) of the enzyme is altered or blocked. The detection of methylation of the same *FMR1* promoter in the grandson favors a role for a trans-acting factor. Not much is known about *de novo* methylation in humans, but it would be worthwhile to test other methylated areas for a general, beneficial methylation defect in these patients.

Finally, our data do not support the hypothesis that methylation of expanded CGG/CCG repeats is necessary for cytogenetic expression of fragile sites. As yet, the molecular basis of four fragile sites (FRAXA, FRAXE, FRAXF, FRA16A) has been characterized as expanded CGG/CCG repeats. These triplets can be methylated, in contrast to the CTG repeat in myotonic dystrophy with a similar repeat length. This latter disorder is not associated with a fragile site and, therefore, methylation was thought to play a key part in cytogenetic expression of these sites. In the two brothers, described in this paper, no methylation of the *FMR1* promoter could be observed, but cytogenetic expression was still evident. The methylation of only a single restriction site was determined and not of the entire repeat, but reports from others (39, 40) have shown that the methylation status of this site can be

extrapolated to the entire area.

After finishing the experiments described in this paper conflicting results were presented by Feng et al. (41). They used fibroblast subclones of a mildly affected patient and showed that despite the presence of normal mRNA levels, transcription is diminished from mRNAs with more than 200 repeats. They concluded that beyond 200 repeats there is no migration of the ribosome along the repeat. However, we show that in a cell line, almost exclusively containing cells with a repeat of 400 CGG copies, translation of *FMR1* mRNA takes place in all cells although at a reduced level compared with controls. Also expression was found in all cells in a blood sample from the same individual (Fig. 3). This expression of the *FMR1* protein in all cells can explain the normal phenotype of this individual.

MATERIALS AND METHODS

Human Subjects

Case 1 (7316). The 14-year-old proband was mentally retarded since childhood, but there was no family history of mental retardation. Physical examination at the age of 14 showed a boy with normal body proportions. No macrocephaly (occipito frontal circumference (OFC) 56 cm (percentile 75-90) and no macrognathia was present. His palate and ears were normal (ear lengths 6,5 cm). He revealed mild hypermobility of distal joints and a soft skin. The external genitalia were small (testes length 2,5 cm) with little pubertal hair development. Psychological testing at the age of 13 years showed a friendly and cooperative boy with good verbal performance. IQ testing at the same age showed a verbal IQ of 64 and a performance IQ of 53 (WISC-R). His behavior was normal without the typical manifestations of fra(X).

Case 2 (7315). The 36-year-old mother of the proband is phenotypically and mentally normal. She studied nursing and is successfully employed in this profession.

Case 3 (7241). The 72-year-old maternal grandfather of the proband is in good health. Physical examination at the age of 72 revealed a normal phenotype. No macrocephaly was present (OFC 57,2 cm; percentile 75) and his forehead was not broad. His ears had lengths of 7,6 and 7,7 cm, but normally shaped. His face was not elongated. No other clinical abnormalities

were found. He refused testicle examination. He has good eye contact and also lacks the other behavioral manifestations of the fra(X) syndrome. He attended high school and became managing-director of a family company of about 100 employees. His mental status could be described as intelligent.

Case 4 (7298). This is the 74-year-old grand-uncle of the proband. His head, face and ears were normally shaped, but he refused detailed physical examination. He had no behavioral manifestations of fra(X). School training was similar to his brother (7241). He was a college student for 3 years after which he became managing-director of the family company. His mental status could also be described as intelligent.

Cytogenetic Studies

Chromosomes were studied from peripheral whole blood lymphocytes. Details of the methodology were described previously (42). Cell cultures were initiated using Chang medium with multiple FRAXA induction systems (FUdR and Thymidine) as described (43). Ten days before harvesting, the long term culture was subcultured on RPMI-1640. A maximum of 200 cells of each sample were examined for the presence of a fra(X) chromosome.

DNA and RNA Studies

Southern blot analysis of DNA, extracted from peripheral blood, was performed according to standard procedures (44) with probe pAO365 (45). The methylation status of the *FMR1* gene was determined by double digests with the methylation sensitive restriction enzyme EagI as has been described before (32). RNA was extracted from EBV transformed cell lines using standard procedures (44). The ratio between HPRT and *FMR1* mRNA was determined, using the protocol of Pieretti et al. (19). Our modifications involve the use of fluorescently labeled PCR primers, followed by analysis on a sequencing gel, using the GeneScanner Software (ABI, Foster City).

Protein Studies

Polyclonal antibodies were raised in rabbits against a GST-*FMR1* fusion protein (α734) (22) and used in immunoprecipitations and Western blotting. Immunoprecipitation, gel electrophoresis and Western blotting were

performed as described (22), with the exception of using ^{125}I protein A instead of alkaline phosphatase conjugated goat anti rabbit IgG to detect the polypeptides. Immunocytochemistry on EBV transformed lymphocytes and on whole blood smears was performed as described (46, 47) using monoclonal antibody 1A (23).

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CHAPTER 10

GENERAL DISCUSSION

Inheritance of Fra(X)

Although there is no doubt that the fra(X) syndrome is an X-linked disease there are two unusual genetic characteristics.

Firstly, an unusual large fraction of the male carriers of the *FMR1* gene is unaffected. Although transmission of the fra(X) through unaffected men is well known (Webb et al., 1981; Fryns and Van den Berge, 1982; Young et al., 1986), the incidence of male transmission has always been underestimated. Extensive family studies have demonstrated grandpaternal transmission in nearly half of the cases (Chapter 2). Apparently, for an X-linked disorder, there is an unusual lack of phenotypic expression in carrier males who are, therefore, called " Normal Transmitting Males" (NTMs). The implications of this phenomenon are tremendous as all daughters of these nonpenetrant males are nonexpressing obligate carriers. However, nearly 40 percent of the grandchildren, male and female, appear to be affected (fig. 1). The "Sherman paradox" refers to these marked differences in penetrance of the mutation in subsequent generations (Sherman et al., 1985).

Secondly, it was found that sibs of NTMs are less likely to be affected than sibs of affected males (Sherman et al., 1985; Smits et al., 1992). These intriguing questions have, to a large extent, been solved in the last few years. This has led not only to a better understanding of the fra(X) syndrome but also to a new genetic concept, the dynamic mutation mechanism.

Repeat Instability in Genetic Disorders

A considerable proportion of the human genome contains repeated DNA. These repeats vary from single bases up to fragments of 40 bases in length (Beckmann and Weber, 1992). The most common simple repeats, defined as microsatellites are dinucleotide repeats, particularly (CA)n (Weber, 1990), which are located at about 50,000 distinct positions throughout the human genome. There are many other such simple tandem repeats, encompassing mono-, tri-, tetra-, and pentanucleotide repeating units (Sutherland and Richards, 1995).

Of these repeats, some trinucleotide repeat stretches appear to be particularly unstable in various genetic disorders. Presently, 8 diseases are associated with triplet repeat expansions (Oostra and Halley, 1995)(fig. 2).

Five folate-sensitive fragile sites (FRAXA, FRAXE, FRAXF, FRA11B and

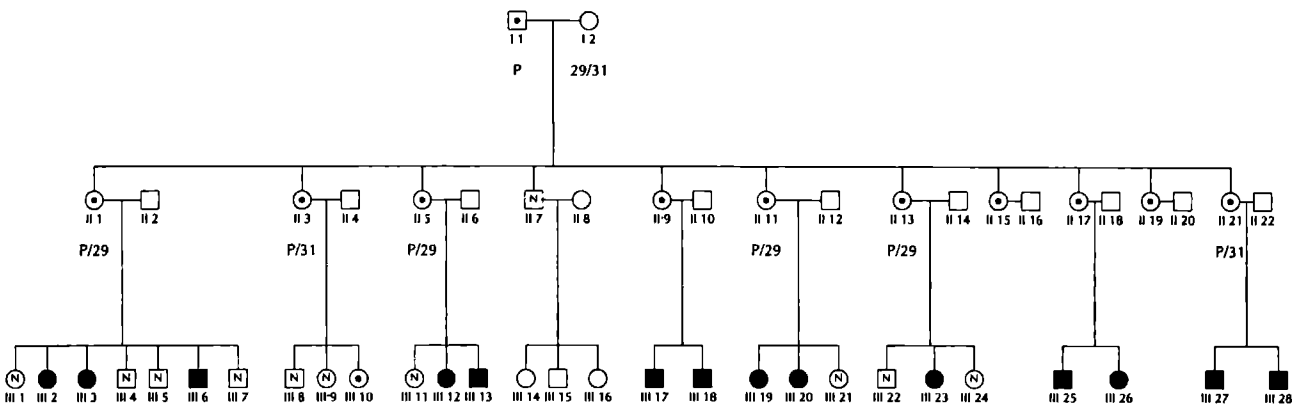

Fig. 1. Pedigree of family 036/FX, showing paternal (I:1) transmission of the *FMR1* gene. Affected individuals are designated by solid symbols; symbols for obligate carrier females with premutations have center circles. "N" in symbol denotes phenotypically normal with CGG repeat length in the normal range. Number under generation number represents the CGG copy number of the grand maternal allele, while "P" indicates the presence of the premutation.

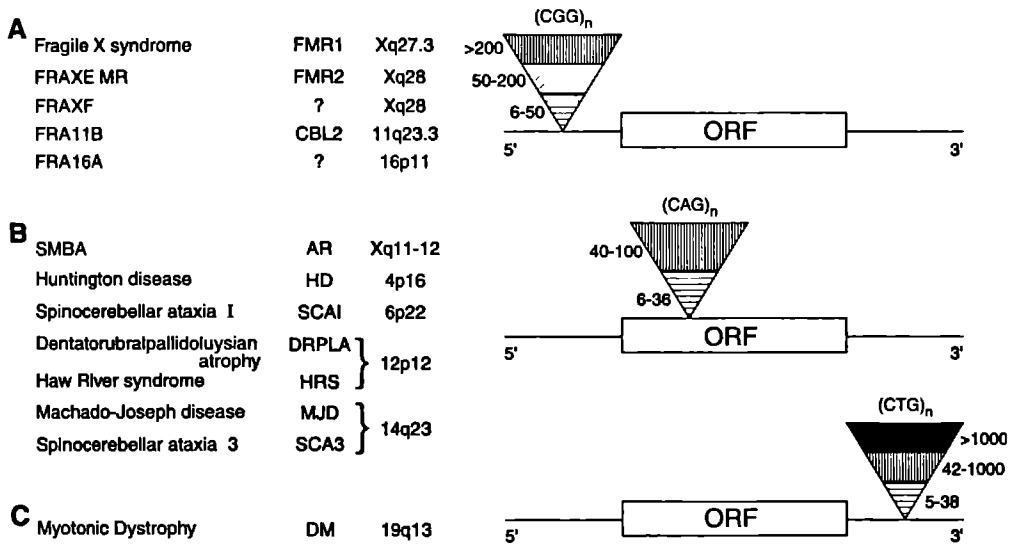

Fig. 2. Schematic diagram of dynamic mutations associated with genetic diseases. Phenotypes with different repeat sizes: (*areas with horizontal lines*) normal; (*area with diagonal lines*) normal (premutation); (*areas with vertical lines*) affected; (*black area*) affected (congenital form). (Adapted from: Oostra and Hally, 1995).

FRA16A) are all composed of polymorphic (CGG) trinucleotide repeats. Studies of FRAXE patients have shown that methylation of CpG residues close to the CGG repeat is associated with mental impairment, similar as seen in fra(X) syndrome (Knight et al., 1993, 1994). However, apart from mild mental retardation, affected males and females did not show a specific phenotype. In addition, some affected FRAXE males have affected daughters (Hamel et al., 1994). The remaining three fragile sites, FRAXF (Hirst et al., 1993), FRA16A (Nancarrow et al., 1994) and FRA11B (Jones et al., 1995), contain hypermethylated and unstable CGG repeats which are not associated with any clinical phenotype. It seems likely that they are not related to the expression of a gene. Jones et al. (1995) suggested an association of a heritable fragile site and

chromosome breakage *in vivo* as they discovered breakage at FRA11B in a number of patients with Jacobson syndrome (11q-).

Studies on FRA16A indicated that methylation might be a consequence of the CGG expansions as the fragile site mutation is in a region of the genome which is normally not methylated (Nancarrow et al., 1994). However, our data show that there is no obligatory relationship between methylation of the *FMR1* gene and CGG repeat expansion (Chapter 9). Thus, additional factors must play a role in the methylation process.

Expansions of (CAG)n trinucleotide repeats, encoding polyglutamine tracts, appear to be associated with five neurodegenerative disorders: Spinal and bulbar muscular dystrophy (SMBA) (Xq11-12) (La Spada et al., 1991), Huntington's disease (4p16) (Huntington's Disease Collaborative Research Group, 1993), Spinocerebellar ataxia type 1 (6p22) (Orr et al., 1993) and type 3 (14q24) (Kawaguchi et al., 1994), Dentorubal-pallidoluysian atrophy (12p12) (Koide et al., 1994). Although the exact mechanism by which such molecular events lead to neurodegeneration is still unknown, it has been suggested that a protein with an expanded polyglutamine tract may lead to a gain of function or to a dominant negative effect which results from aberrant interaction with itself, its normal target or a new gene product (Banfi et al., 1994).

Myotonic dystrophy was the first autosomal disease identified by amplification of a trinucleotide repeat. Amplification of the CTG tract (equivalent to CAG) above the threshold length of 38 repeats results in abnormal mRNA stability (Fu et al., 1993; Sabouri et al., 1993) and the extremes of the clinical phenotype correlates with the size of the mutation (Brook et al., 1992; Harley et al., 1992; Hunter et al., 1992). Animal equivalents of the trinucleotide repeats involved in human disease encode shorter length repeats, a greater stability and lower degree of polymorphism which suggests that the mechanism of dynamic mutations may largely be restricted to the human genome (Sutherland and Richard, 1995).

Microsatellite instability has also been noted as a frequent genetic alteration in human cancer cells (Ionov et al., 1993; Thibodeau et al., 1993; Peltomaki et al., 1993; Wooster et al., 1994). Instability of mono-, di- and trinucleotide repeats appear to be associated with various inherited cancer syndromes (reviewed by Richards and Sutherland, 1994). Tract instability of simple repeats may be a consequence of either an increased rate of DNA polymerase slippage or a decreased efficiency of mismatch repair (Strand et al., 1993).

Genetic Background and CGG Repeat (In)stability

How and how often fra(X) premutations arise in the general population is still largely unknown. Haplotype analyses with flanking (CA)n markers have revealed linkage disequilibrium between fra(X) and normal X chromosomes, which indicates a limited number of predisposing normal alleles (Richards et al., 1992; Smits et al., 1993; Macpherson et al., 1994).

We have searched for evidence of paternal or maternal genetic components outside the *FMR1* gene, which might predispose for repeat instability. If paternal factors contributed to the degree of expansion of the maternally transmitted CGG repeat, length variation of the repeat between halfsibs, having different fathers, would be expected. To assess the paternal contribution to repeat expansion, we have analyzed fifteen half sisters/brothers in five families (fig. 3). Comparison of the CGG repeat length between the halfsibs demonstrated either a premutation or a full mutation in all the paired samples, independent of the paternal inheritance (Smits et al. unpublished data). These results indicate that only maternal factors influence CGG repeat stability.

It could also be speculated that the non-mutated X chromosome in fra(X) carrier females might influence the stability of the CGG repeat. Heterozygotes were, therefore, compared according to the maternal origin of the non-mutated X chromosome in sisters carrying the paternally inherited premutation. We found in two out of six obligate carriers (fig. 1, II:3 and II:21) that expansion from a premutation to a full mutation in the offspring did not occur, irrespective of which maternal inherited X chromosome was present (Smits et al. unpublished observations). Recent evidence suggests that the instability probably is a function of the composition of the trinucleotide repeat. The *FMR1* trinucleotide repeat array is a compound structure of (CGG)n with some interruptions by AGG triplets at the 5' end of the repeat, while the 3' end shows the length variations with the longest arrays of perfect CGG repeats. Sequence analysis of normal alleles revealed haplotype-specific variation of the repeat (Kunst and Warren, 1994; Hirst et al., 1994). Alleles in the normal size range but having at least 24 perfect CGG repeats in the 3' end appear more frequently on haplotypes overrepresented among fra(X) chromosomes. Similar data were found upon sequence analysis of premutations, 63 percent had no interruptions of AGG triplets, while 37 percent had only one AGG (Zhong et al., 1995). These data indicate that the stability of repeats in the normal range may be dependent on the nature of the interspersed AGG triplets.

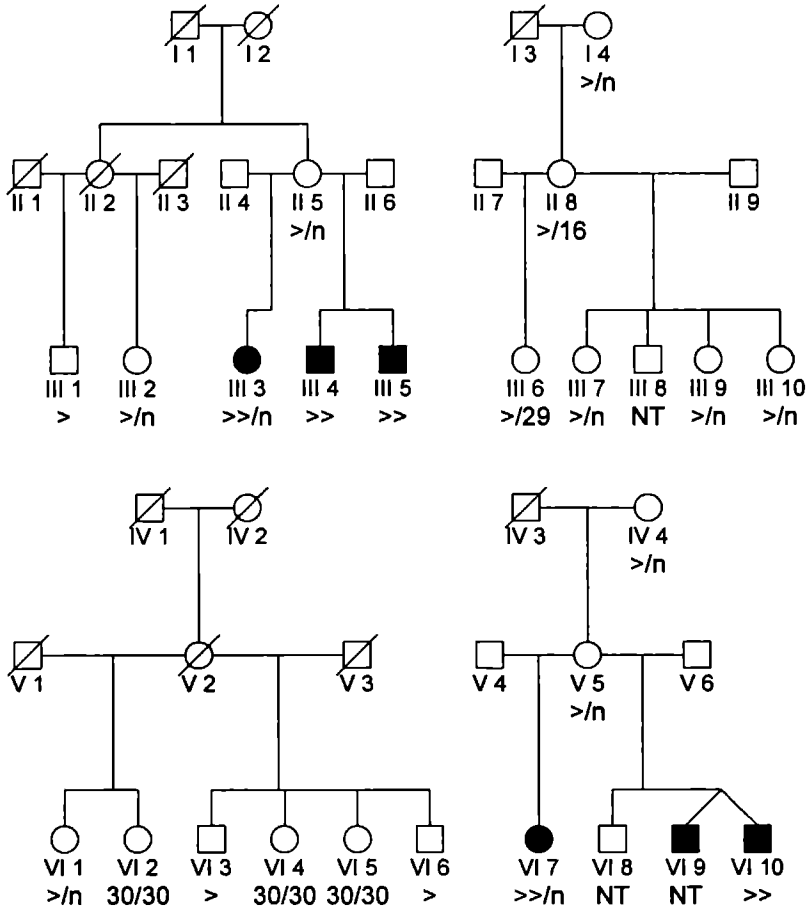

Fig. 3. Pedigrees with halfsibs having different fathers. Solid symbols denote affected individuals. The presence of the full mutation, premutation and a normal sized *FMR1* gene as analysed by Southern blotting are indicated by ">>", ">", and "n" respectively. The arabic numbers refer to the length of the CGG fragment as determined by PCR.

Diagnostic Issues of the Fra(X) Syndrome

The discovery of the molecular basis of the fra(X) syndrome has had a major impact on the diagnosis of the disease. The vast majority of fra(X) patients appear to have a dramatic increase in length of the CGG repeat at the 5' end of the *FMR1* gene. However, some patients have been described who showed

typical clinical features of the fra(X) syndrome without amplification of the CGG repeat and/or cytogenetic expression. In these cases the patients appeared to have a partial *de novo* deletion (Gedeon et al., 1992; Wöhrle et al., 1992; Tarleton et al., 1993; Gu et al., 1994; Trottier et al., 1994; Lugenbeel et al., 1995; Quan et al., 1995), a familial deletion (Meijer et al., 1994) or a point mutation in the *FMR1* gene (De Boulle et al., 1993). All these alterations resulted in silencing of the *FMR1* gene with the same phenotypic consequences. Although the incidence of *de novo* deletions seems to be low, we have identified a family with two affected brothers (fig. 4, II:4 and II:5) both carrying a deletion of the CGG repeat, whereas an other mentally impaired relative exhibits an amplification of the CGG repeat (II:2). Linkage analysis was compatible with the mutated X chromosome being the same in both family branches (Smits et al. unpublished observations). These data suggest that the deletion of the CGG repeat is secondary to its amplification, in line with previous observations in 4 patients all expressing a full mutation besides the presence of a deletion (De Graaff et al., 1995).

That the fra(X) syndrome may be caused by amplification of the CGG repeat, deletion of the CGG repeat, or a point mutation has the practical consequence that no single DNA analysis technique is sufficient to detect all the possible mutations. As the point mutations appear to be very rare, DNA sequence analysis is only carried out if there are very convincing clinical signs. For screening purposes, PCR analysis of the CGG repeat is widely used. This method will detect amplifications and deletions of the CGG repeat in males. This has to be followed up by Southern blot analysis to distinguish between the two latter possibilities. If in females only 1 PCR fragment is seen, Southern blot analysis has to be performed also, to establish whether or not two normal sized alleles are present.

We have shown (Chapter 9) that methylation of the promoter region inhibits expression of the *FMR1* gene and that repeat expansion itself is not sufficient for expression of the fra(X) phenotype. In case of a discrepancy between the mental status and the CGG repeat length, the methylation status should be assessed. Unfortunately, methylation tests are not useful for prenatal diagnosis. Iida et al. (1994) have compared the extent of methylation in fetal brain, cord blood, chorionic villi and placenta and found a discrepancy between embryonic and extra-embryonic tissues. These observations are in agreement with those of Sutherland et al. (1991) and Sutcliffe et al. (1992) who showed that villous samples do not exhibit the ultimate methylation state. Indeed it has been found that chorionic villi of males with a full *FMR1* mutation, taken before the 11th week of gestation, may show an unmethylated DNA pattern (Oostra, personal

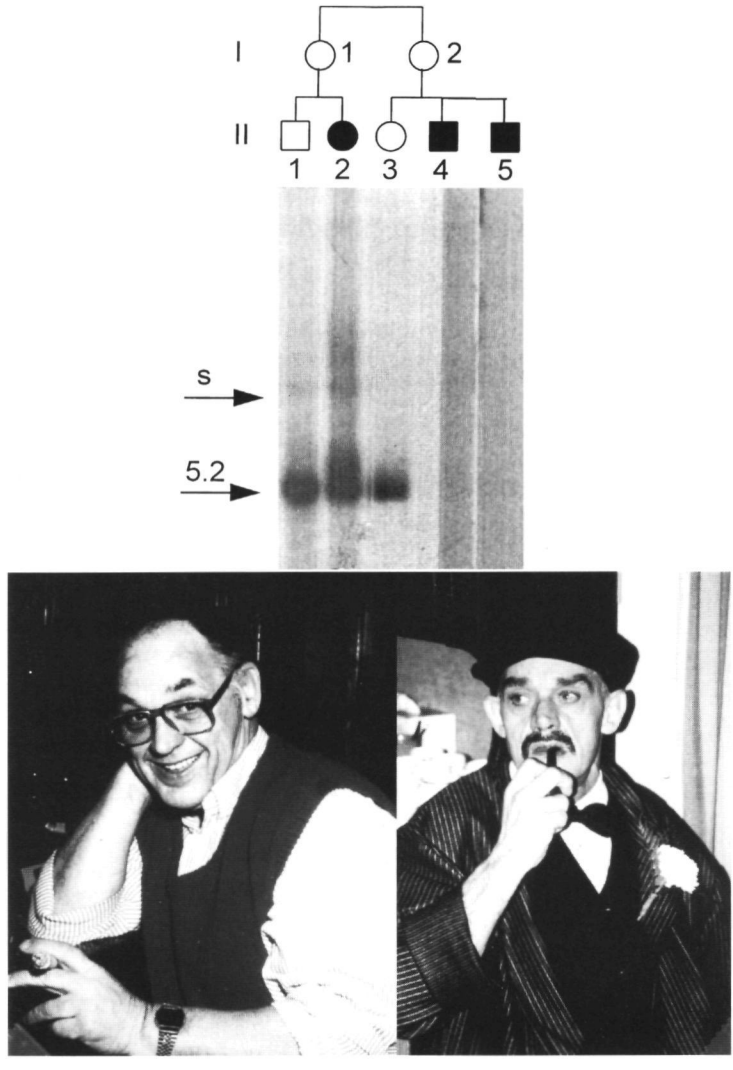

Fig. 4. Affected brothers (II:4 and II:5) are both carrying a deletion of the CGG repeat in the *FMR1* gene. Southern blot analysis of genomic DNA digested with EcoRI and hybridized with probe PAO365 showed the familial deletion (lane 4 and 5) as well as an expansion (lane 2) of the CGG repeat in the *FMR1* gene. 5.2 kb shows a fragment of normal length. S= dispersed segment from which this smear developed upwards.

communication). Recently, an antibody test on bloodsmears for diagnosing fragile X syndrome was developed (Willemsen et al., 1995). This test is based on presence or absence of FMRP (*FMR1* Protein). A similar test on chorionic villi for prenatal diagnosis of fragile X syndrome is validated. An example of this test is shown in figure 5.

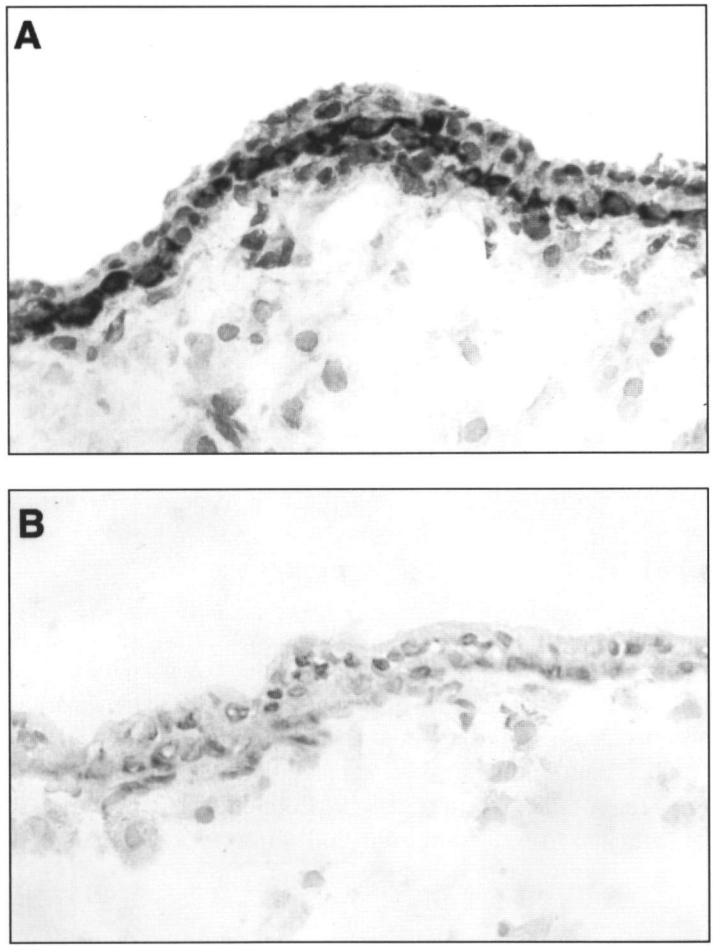

Fig. 5. FMRP expression in chorionic villi (c.v). using the indirect immunoperoxidase technique on cryostat sections. A = c.v. (12.5 weeks) control fetus. B = c.v. (12.5 weeks) affected male fetus. The dark staining in the cytotrophoblast cells from control villi (A) indicates the presence of FMRP, whereas in the cytotrophoblast cells from the affected male fetus (B) no FMRP could be observed. (FMRP test provided by Dr. R. Willemsen).

Several tissues, including brain, of this male fetus did not express any FMRP (Oostra, personal communication). More studies on fetal cells in case of termination, and in cord blood/peripheral blood after birth, should be done to support this preliminary result.

Benefits of Active Genetic Counseling in Fra(X) Families

Relatives of identified fra(X) syndrome patients should be informed about genetic risk factors and, subsequently, given access to carrier-testing. It is important to note that usually most individuals are not aware of the presence of a fra(X) mutation in their family. Therefore, it is an important task for the genetic counseling clinic to perform systematic and careful active genetic counseling. According to this active approach, we have studied 100 multigenerational fra(X) families in the past fifteen years. The mutation could be deduced or directly detected in more than 700 carriers. Excluding the index patients, 480 female carriers (150 affected) and 220 male carriers (170 mentally retarded and 50 NTMs) were identified (unpublished observations). Previously undiagnosed mentally retarded individuals in these families could readily be diagnosed. An early diagnosis does not only prevent unnecessary medical investigations but also makes a timely start of a therapeutic strategy possible. Particularly, physicians should be in a position to apply appropriate therapies which may include medication, language and occupational therapy, counseling and special education, as needed (Hagerman et al., 1992).

For parents of fra(X) patients and their relatives, it is extremely important to have the opportunity to make a reproductive choice based on adequate information concerning diagnosis, prognosis, recurrence risk and the availability of post- and prenatal diagnosis. As long as they are informed, the primary aim of active genetic counseling has been achieved. The secondary effects of genetic counseling of females at risk on decisions about childbearing may lead to the use of prenatal tests and to reduction in birth of affected individuals (Turner et al., 1992).

Since 1991, we performed prenatal tests, based on direct detection of the CGG repeat length, in 25 cases using chorionic villus sampling at the end of the first trimester of pregnancy. A full *FMR1* mutation was found in 10 cases and in all of them the parents opted for termination of the pregnancy. The decision whether or not to terminate was (and still is) very difficult, in particular, in case of a female fetus with a full mutation, as the incomplete penetrance has to be taken into account. As long as it is impossible to accurately predict whether and

to which degree a female fetus with a full mutation will be affected, this question will remain among the most difficult ones for all families concerned (Fryns, 1989).

Population Based Screening for Fra(X) Syndrome

As the population frequency of the fra(X) gene is quite high, different scenarios for population based screening programs have been proposed. Screening of all pregnant women would, in theory, be a comprehensive approach of carrier detection for the fra(X) syndrome. Apart from ethical considerations and practical limitations, the main arguments against screening of all pregnant women are (i) that precise prevalence figures for the premutation in the population are lacking and (ii) that there is a lack of accurate information about the overlap (grey zone) between extended normal alleles and small premutations. Although there is evidence that the prevalence of premutations might be high (Rousseau et al., 1993, 1995; Reiss et al., 1994), it is proposed by the American College of Medical Genetics (ACMG) (1994) that additional information on the repeat stability has to be obtained from populations unselected for the fra(X) syndrome. We agree, as stated by ACMG (1994), that screening of pregnant women is not indicated until these matters have been clarified.

A less controversial approach would be to search for full mutations in newborn males, as was suggested by Young (1993). Direct detection of the CGG repeat length is very accurate and has the advantage that an accurate prognosis can be given, supplemented by the offer to investigate the extended family. However, this DNA test is still labour-intensive, technically demanding and expensive to perform on a large scale basis (Bonthron and Strain, 1993). An alternative assay could be to test the presence or absence of the gene product FMRP (Oostra and Halley, 1995). This rapid antibody test, suitable for screening of large numbers of individuals, may be easy to combine with e.g. the PKU (phenylketonuria) test.

The ACMG (1994) proposed that fra(X) screening should be considered for individuals of either sex with mental retardation, developmental delay or autism, especially if they have (a) any physical or behavioral characteristics reminiscent of fra(X) syndrome, (b) a family history suspect for fra(X) syndrome or (c) male or female relatives with undiagnosed mental retardation. Several studies on selected populations of mentally retarded individuals have confirmed that fra(X) patients account for a substantial proportion of residential patients (Blomquist et al., 1982, 1983; Bundey et al., 1985; Kähkönen et al., 1986; Fryns, 1989). In

a study of 350 mentally retarded institutionalized adult males we found 10% positive for the fra(X) syndrome thus far. Figure 6 gives an idea of the variety of clinical features of patients in such an institute.

Fig. 6. Picture of institutionalized adult males, showing a large variety of clinical features. Three of them were identified as having fra(X) syndrome.

Among this latter group, 5 persons were selected for fra(X) repeat testing based on clinical features only and, subsequently, three of them were identified as having the fra(X) syndrome.

In the Netherlands about 125000 individuals are mentally impaired, including 35000 institutionalized patients (NVGZ, 1995). Fryns et al. (1984) proposed that nearly 3 percent of the total mentally retarded population may be attributed to the fra(X) syndrome. Supporting evidence for the high prevalence of the fra(X) syndrome came from Webb et al. (1986, 1991) and Turner et al. (1986, 1992). In a population of 1.2 million in Australia, 2000 intellectually handicapped persons were screened. The estimated prevalence rates for the fra(X) syndrome with all degrees of mental impairment were 1:2600 for males and 1:4200 for

females. Based on these estimations, approximately 4500 fra(X) patients can be predicted for the Netherlands. Up to now, less than 500 of all fra(X) male patients have been diagnosed (Oostra, in preparation). With regard to this result, the recommendation can be made that a preventive screening program in mentally retarded populations should be performed in order to identify all fra(X) patients. Every identified fra(X) index patient may serve as a starting-point for active genetic counseling of relatives at risk.

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SAMENVATTING

Mentale retardatie, ofwel een verstandelijke handicap, komt relatief vaak voor. In Nederland zijn ongeveer 125000 mensen verstandelijk gehandicapt waarvan er vermoedelijk 4500 lijden aan het zogenaamde fragiele X (fra(X)) syndroom. Naast mentale retardatie kent het fra(X) syndroom ook enkele niet specifieke lichamelijke kenmerken zoals vergrote testikels, een lange smalle gelaatsvorm en grote afstaande oren. Kenmerkend is ook het typische taal- en spraakgebruik met een betekenisloze herhaling van vooral vragende taaluitingen. Bovendien vertonen veel patiënten een overbeweeglijk gedrag, ze hebben concentratiemoeilijkheden en ze zijn schuw in sociale situaties, waarbij het vermijden van oogcontact opvalt.

Het fra(X) syndroom dankt zijn naam aan een fragiele plaats op het uiteinde van de lange arm van het X chromosoom. Al in 1969 werd ontdekt dat er een verband bestaat tussen het voorkomen van deze fra(X) plaats en mentale retardatie. Nadat bijna 10 jaar later bekend was geworden dat een laag foliumzuurgehalte bij het kweken van cellen vereist is om deze fragiele plaats licht-microscopisch zichtbaar te maken, kon diagnostiek op basis van chromosomenonderzoek en vervolgens erfelijkheidsvoorlichting in fra(X) families worden verricht. Diagnostiek door middel van chromosomenonderzoek bleek echter in bepaalde gevallen niet betrouwbaar. Ofschoon fra(X) patiënten wel goed detecteerbaar waren bleek dragerschap bij gezonde personen niet of moeilijk vast te stellen.

Sinds 1986 werd naast het chromosomenonderzoek ook gebruik gemaakt van DNA-koppelingsonderzoek. Hiermee kon indirect de waarschijnlijkheid dat het fra(X) gen aanwezig was bepaald worden zonder dat het betrokken gen zelf bekend was. Dit was mogelijk door kleine variaties in het DNA (merkers) te gebruiken die in de directe nabijheid van het fra(X) gen waren gelegen en die duidelijk samen met het ziektebeeld overerfden in de familie. Hierdoor verbeterde de betrouwbaarheid van de bestaande onderzoeksmethode aanzienlijk en vals negatieve uitslagen op basis van het chromosomenonderzoek konden veelal (tijdig) worden gecorrigeerd.

In 1991 leidde moleculair genetisch onderzoek tot de isolatie van het *FMR1* gen (fragiele X mentale retardatie). Aan het begin van dit *FMR1* gen werd een specifieke basevolgorde aangetroffen bestaande uit een repeterende volgorde van drie DNA bouwstenen (CGG-triplet (C(Cytosine) G(Guanine) G(Guanine)). Het *FMR1* gen bevat in normale situaties een aantal van 6 tot 50 CGG-triplets. Mutaties in het *FMR1* gen blijken het gevolg te zijn van een (sterk) toegenomen aantal van deze CGG-triplets. Er zijn twee typen *FMR1* gen mutaties te onderscheiden: (i) een kleine verandering (premutatie) met een lengte tussen 50-200 CGG-triplets. Deze heeft geen klinische consequenties, (ii) een grote verandering (volledige mutatie) waarbij aanzienlijk meer dan 200 CGG-triplets voorkomen. Dit grote aantal CGG-

triplets leidt vrijwel altijd tot inactivatie van het *FMR1* gen. Hierdoor komt het *FMR1* proteïne (FMRP), waarvoor dit gen codeert en dat van belang is voor een normale verstandelijke ontwikkeling, niet of maar gedeeltelijk tot expressie. Dit heeft het fra(X) fenotype (klinische verschijnselen passend bij het fra(X) syndroom) tot gevolg. Nadat de lengte van het betreffende deel van het *FMR1* gen, dat direct afhankelijk is van het aantal CGG-triplets, nauwkeurig kon worden bepaald werd het mogelijk deze als directe diagnostische test toe te passen. De sensitiviteit (percentage zieke personen met een positief testresultaat) van de directe DNA test bleek zeer hoog zowel bij mannen als vrouwen. De specificiteit (percentage niet-zieke personen met een negatief testresultaat) van deze test bij vrouwen was echter matig omdat een belangrijk deel van de vrouwen met een volledige fra(X) mutatie mentaal normaal functioneerde. Derhalve was de voorspellende waarde bij prenatale diagnostiek, in geval van een vrouwelijke foetus met een volledige mutatie, beperkt.

De ontdekking van de verschillende typen mutaties in het *FMR1* gen verklaarde ook de etiologie en het bijzondere overervingspatroon van het fra(X) syndroom. Het was opmerkelijk dat de hoge incidentie van het fra(X) syndroom in de bevolking niet verklaard kon worden door het frequent ontstaan van nieuwe mutaties. In alle bestudeerde families kon de aanwezigheid van een premutatie in het *FMR1* gen steeds in voorafgaande generaties worden aangetoond. Door de overerving van een nauw gekoppelde genetische merker te bestuderen, in fra(X) families die onderling verwant waren, bleek dat de fra(X) mutatie gedurende vele generaties voorkwam. Er zijn zelfs aanwijzingen dat fra(X) patiënten in meerderheid afstammen van een relatief kleine groep gemeenschappelijke voorouders. Wat de plotselinge toename van het aantal CGG-triplets bij patiënten veroorzaakt is niet met zekerheid vastgesteld. Het lijkt waarschijnlijk dat over meerdere generaties kleine veranderingen optreden in de repeterende CGG-volgorde en lengte van het *FMR1* gen, waardoor de stabiliteit van dit DNA-fragment afneemt.

Het overervingspatroon van de fra(X) mutatie bleek in sterke mate bepaald te worden door het geslacht van de ouder. Mannelijke dragers van een premutatie gaven deze premutatie vrijwel onveranderd door aan dochters. Deze dochters vertoonden evenals hun vaders geen fra(X) fenotype, maar bleken zelf wel zonen en dochters te hebben met een premutatie dan wel een volledige mutatie. De penetrantie (frequentie waarmee bij dragers van de mutatie het verwachte fenotype tot uiting komt) was bij zonen 85%. Deze incomplete penetrantie kon verklaard worden door de aanwezigheid van premutaties. De penetrantie bij dochters bleek een veelvoud te zijn van wat literatuurgegevens vermeldden, namelijk 64%. Deze beduidend lagere penetrantie dan bij zonen werd veroorzaakt doordat er naast dochters met een premutatie ook dochters voorkwamen met een volledige fra(X) mutatie zonder dat dit leidde tot het fra(X) fenotype. Dit lijkt verband te houden met het proces van lyonisatie (naar willekeur wordt in alle cellen één van de twee

X-chromosomen geïnactiveerd). Als in de meeste cellen het X-chromosoom met een volledige mutatie in het *FMR1* gen geïnactiveerd werd had de aanwezigheid van een volledige fra(X) mutatie geen nadelige gevolgen voor de mentale ontwikkeling. In gezinnen waarin de moeder draagster was van een volledige fra(X) mutatie werd de hoogste penetrantie vastgesteld. Over het algemeen vertoonden meisjes in vergelijking met jongens met het fra(X) syndroom een minder grote ontwikkelingsachterstand die zich beperkte tot voornamelijk leermoeilijkheden.

Een wetenschappelijk interessante bevinding was de totale afwezigheid van het fra(X) fenotype bij 2 broers met een volledige fra(X) mutatie. Deze situatie toonde aan dat er geen absolute relatie bestaat tussen het aantal toegenomen CGG-triplets en de inactivatie van het *FMR1* gen. Expressie van het FMRP bleek ook bij aanwezigheid van een volledige fra(X) mutatie plaats te kunnen vinden en cruciaal te zijn voor een normale mentale ontwikkeling. Een recent ontwikkelde diagnostische test maakt het thans mogelijk om op relatief eenvoudige en snelle wijze de aan- of afwezigheid van het FMRP vast te stellen.

Tenslotte heeft het hier beschreven onderzoek aangetoond dat de primaire doelgroep voor onderzoek naar het voorkomen van de fra(X) mutatie mentaal geretardeerden zijn. Mede door de intensieve samenwerking met artsen die werkzaam zijn in de zwakzinnige zorg kon bij ruim 100 index patiënten het fra(X) syndroom worden gediagnostiseerd. Door familieleden van deze fra(X) patiënten actief te benaderen om hen erfelijkheidsvoorlichting te geven konden velen vroegtijdig op de hoogte gebracht worden van een mogelijk risico op kinderen met het fra(X) syndroom. Vrijwel iedereen stelde onderzoek naar het voorkomen van drager-/draagsterschap van de fra(X) mutatie op prijs. Bij ruim 700 familieleden van fra(X) patiënten werd door middel van DNA-onderzoek drager-/draagsterschap aangetoond of afgeleid. DNA-diagnostiek bood ook voldoende zekerheid om aan draagsters prenatale diagnostiek aan te bieden. Voor een aantal draagsters van een fra(X) mutatie was de optie van prenatale diagnostiek met de mogelijkheid van selectieve abortus aanvaardbaar. In deze sterk belaste families konden echter ook zeer velen gerustgesteld worden ten aanzien van een verhoogd risico op kinderen met een verstandelijke handicap. Bij hen werd op basis van betrouwbaar diagnostisch onderzoek drager-/draagsterschap van de familiair voorkomende fra(X) mutatie uitgesloten.

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Rest mij nog hen te bedanken die de meest wezenlijke bijdrage aan mijn onderzoek hebben geleverd, namelijk de fragiele X patiënten en hun families.

CURRICULUM VITAE

Arie Smits werd geboren op 7 juni 1947 in Beuningen. Na het beëindigen van Land en Tuinbouw opleidingen (LTS, 1964 en RMTS, 1967) vervulde hij gedurende 2 jaren zijn militaire dienstplicht bij het Korps Mariniers. In april 1971 werd hij aangesteld als cytogenetisch analist bij het Anthropogenetisch Instituut van de Universiteit te Nijmegen. In 1972 behaalde hij het HAVO diploma aan de Avondscholengemeenschap "Craneveldt" te Nijmegen. Na enkele jaren werd hij in toenemende mate betrokken bij erfelijkheidsvoorlichting en onderzoek in families waarin chromosomale veranderingen voorkomen. Ter ondersteuning en verdieping van het familieonderzoek werden achtereenvolgens met succes opleidingen gevolgd ter verkrijging van een tweedegraads lesbevoegdheid in de Ortho-Pedagogiek (M.O.-A, Hoogveld Instituut te Nijmegen, 1979) en een eerstegraads lesbevoegdheid in de Sociale Pedagogiek (M.O.-B, Universiteit te Nijmegen, 1984). Aan dezelfde Universiteit werd in 1987 het doctoraal-diploma behaald in de studierichting der Andragogische Wetenschappen met als bijvakken Empirische Pedagogiek en Medische Sociologie. Aansluitend aan deze studie werd hij stafmedewerker bij de sectie Cytogenetica. Vanaf 1981 verricht hij systematisch onderzoek in fragiele X families. De resultaten hiervan liggen ten grondslag aan dit proefschrift. Sinds 1992 neemt hij ook deel aan onderzoek naar het opsporen van genen die verantwoordelijk zijn voor geslachtsgebonden mentale retardatie in families. Arie Smits is gehuwd met Ans Sonneveld en heeft 2 dochters: Femke en Marieke.

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STELLINGEN

behorend bij het proefschrift

"The fragile X syndrome: genetic and diagnostic aspects"

1. Er is geen direct verband tussen de lengte van de CGG repeat in het *FMR1* gen en het fragiele X fenotype (*Dit proefschrift*).

2. Systematisch onderzoek in fra(X) families naar dragerschap van mutaties in het *FMR1* gen heeft de voorkeur boven populatiescreening (*Dit proefschrift*).

3. De voorspellende waarde van de directe DNA-test, bij een vrouwelijke foetus met een volledige fra(X) mutatie, is beperkt (*Dit proefschrift*).

4. Door het foundereffect bij het fra(X) syndroom zijn nagenoeg alle dragers/draagsters van *FMR1* gen mutaties in Nederland op te sporen.

5. Non-directieve erfelijkheidsadvisering aan verstandelijk gehandicapten met kinderwens stelt de counseler voor een dilemma.

6. Meer inspanning vanuit de klinische genetica, om familieleden "at risk" tijdig te informeren, zou veel ernstig leed kunnen voorkomen.

7. In the strictest sense of the word dynamic mutations are not really mutations, but new and different mechanisms that result in disease (Hall, 1994 Growth Genetics & Hormones: Vol. 10 No.4:11).

8. Intellectual handicaps are costly both emotionally and financially, and the costs span a life time (Turner et al., 1986; New England Journal of Medicine: 315:607-609).

9. Een hoge maternale leeftijd vergroot de kans op het krijgen van een gezond kind, wanneer er sprake is van een mannelijke drager van een gebalanceerde chromosomale translocatie t(21q21q) of t(22q22q).

10. Bij het spelen van tennis biedt het net de meeste tegenstand.

Nijmegen, 18 april 1996 **A.P.T. Smits**